Highlights

- Relative importance of (a)biotic controls of methane oxidation was determined.
- Edaphic properties seem to be more important in regulating methane oxidation.
- Initial composition determines the trajectory of methanotroph succession.
- Type II methanotrophs were favored during re-colonization.

Disentangling abiotic and biotic controls of aerobic methane oxidation during re-colonization.

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Abstract

Aerobic methane oxidation is driven by both abiotic and biotic factors which are often confounded in the soil environment. Using a laboratory-scale reciprocal inoculation experiment with two native soils (paddy and upland agricultural soils) and the gamma-irradiated fraction of these soils, we aim to disentangle and determine the relative contribution of abiotic (i.e., soil edaphic properties) and biotic (i.e., initial methanotrophic community composition) controls of methane oxidation during re-colonization. Methane uptake was appreciably higher in incubations containing gamma-irradiated paddy than upland soil after inoculation with both native soils despite of different initial methanotrophic community composition, suggesting an overriding effect of the soil edaphic properties in positively regulating methane oxidation. Community composition was similar in incubations with the same starting inoculum as determined by quantitative and qualitative *pmoA* gene analyses. Thus, results suggested that the initial community composition affects the trajectory of community succession to an extent, but not at the expense of the methanotrophic activity under high methane availability; edaphic properties override initial community composition in regulating methane oxidation.

48 *1.0 Introduction*

49

50 Aerobic methane-oxidizing bacteria (methanotrophs) represent a specialized microbial guild
51 characterized by their ability to use methane as a carbon and energy source. Aerobic
52 methanotrophs belong to *Verrucomicrobia* and *Proteobacteria*, with members of the
53 proteobacterial methanotrophs falling within the classes *Gammaproteobacteria* (comprising
54 of subgroups type Ia and Ib) and *Alphaproteobacteria* (subgroup type II). While the
55 verrucomicrobial methanotrophs were discovered in low pH (< 5) and high temperature (>
56 50^{o}C) geothermal environments, the proteobacterial methanotrophs are widespread, but
57 show habitat specificity (Op den Camp HJM et al., 2009; Knief et al., 2015). Methanotrophs
58 possess the enzyme methane monooxygenase that enables them to oxidize methane to
59 methanol, the initial step in methane oxidation (Semrau et al., 2010). Typically, the structural
60 genes encoding for the soluble and particulate form of the methane monooxygenase
61 enzyme (*mmoX* and *pmoA*, respectively) are targeted to survey the methanotrophic diversity
62 in complex microbial communities (Wen et al., 2016). In wetland ecosystems,
63 methanotrophs inhabit oxic-anoxic interfaces with oxygen-methane counter-gradients (e.g.,
64 soil-overlaying water, aquatic plant roots) where they act as a filter to consume methane
65 produced in the deeper anoxic sediment layers (Reim et al., 2012). On the other hand,
66 methanotrophs in well-aerated upland soils serve as a methane sink, consuming
67 atmospheric methane (Kolb et al., 2009; Shrestha et al., 2012; Ho et al., 2015a; 2019;
68 Pratscher et al., 2018). In both these roles, abiotic (e.g., substrate concentrations,
69 micronutrients, and other soil physico-chemical properties; Hütsch et al., 1994; Bodelier
70 2011; Veraart et al., 2015; Ho et al., 2013; 2018; Semrau et al., 2018) and biotic factors (e.g.,
71 methanotrophic community composition/abundance and interaction-induced response in

72 community functioning; Ho et al., 2016a; Malghani et al., 2016; Chang et al., 2018; Reumer
73 et al., 2018; Schnyder et al., 2018; Veraart et al., 2018) are known to drive aerobic methane
74 oxidation. Collectively, we refer to the non-biological attributes inherent to the soil as abiotic
75 parameters, whereas biotic determinants are exemplified by the initial methanotrophic
76 community composition.
77
78 Soil manipulation (e.g., amendment) studies, and experimental design capitalizing on soil
79 chronosequence with natural environmental gradients are typically used to relate changes in
80 (a)biotic factors to community functioning (e.g., Rousk et al., 2010; Bissett et al., 2012; Ho et
81 al., 2013; 2018; Palmer & Horn, 2015; Shiau et al., 2018). Often, the abiotic and biotic
82 determinants are confounded, obscuring the contribution of either factors to the regulation
83 of methane oxidation. Rarely are there factors explicitly tested independently and
84 simultaneously.
85
86 Here, we aim to disentangle abiotic and biotic controls of methane oxidation by employing a
87 reciprocal inoculation experimental design using two native soils and the gamma-irradiated
88 (25 kGy; ^{60}Co) fractions of these soils (Figure 1), which enabled us to relate the (a)biotic
89 determinants to methane oxidation. We anticipate that if (i) abiotic determinants exert a
90 stronger control than biotic determinants in regulating methane oxidation, the same abiotic
91 environment (i.e., gamma-irradiated soil) will consistently support high methane uptake
92 regardless of the initial community composition, (ii) biotic determinants exert a stronger
93 control than abiotic determinants, the same native soil harboring the initial methanotrophic
94 community will consistently exhibit high methane uptake regardless of the edaphic
95 properties, and (iii) there is no consistent effect, less predictive and/or stochastic factors

(e.g., priority effect, site history) may have an overriding impact on the contemporary methanotrophic activity. To address our suppositions, we incubated oxic soil microcosms containing native and gamma-irradiated native soils in all combinations (Figures 1) for 35 days, and followed the potential methane oxidation rate over time (Figure 2). Two soils (wetland paddy and upland agricultural soils) with distinct physico-chemical properties and different methanotrophic communities with proven methane uptake capacity under high methane availability (> 2 $\%_{v/v}$) were used (Table 1; Ho et al., 2013; 2015a). Soil processing, and soil microcosm setup and sampling are detailed in the Supplementary Materials.

The temporal succession of the methanotrophic community composition was monitored during the incubation using group-specific qPCR assays (i.e., MBAC, MCOC, and TYPEII assays targeting the methanotroph subgroups type Ia, Ib, and II, respectively; Kolb et al., 2003; Ho et al., 2016b; Supplementary Materials) and Illumina MiSeq sequencing of *pmoA* gene amplicons. The *pmoA* gene, instead of the *mmoX* gene, was targeted because the *mmoX* gene transcript or the methanotrophs harbouring only the *mmoX* gene were not detected or below the detection limit in these soils (Reim et al., 2012; Ho et al., 2015a). While the group-specific qPCR assays were performed to be used as proxies for methanotroph abundances, high throughput sequencing of the *pmoA* gene was performed to follow compositional changes in the methanotrophic community. The *pmoA* gene sequences were analysed as described before (Reumer et al., 2018; Supplementary Materials), and were deposited at the NCBI Sequence Read Archive under the accession number SRR9924748 (NCBI BioProject PRJNA559227).

2.0 Abiotic parameters exert a stronger effect on aerobic methane oxidation than the initial methanotrophic community composition.

The potential for methane oxidation (total methane consumed) was consistently significantly higher in the microcosms containing the gamma-irradiated paddy soil (*PP*, 190 ± 15 µmoles g dw^{-1}; *UP*, 231 ± 42 µmoles g dw^{-1}) than those containing the gamma-irradiated upland soil (*PU,* 66 ± 26 µmoles g dw^{-1}; and *UU*, 64 ± 17 µmoles g dw^{-1}), regardless of the initial community composition (Figure 2). This suggests that the abiotic rather than the biotic determinants, more strongly regulated methane oxidation, which is in line with our first supposition. Higher methanotrophic activity in microcosms containing gamma-irradiated paddy than upland soil coincided with the higher initial ammonium concentration (paddy soil, 3.4 – 4.2 µmoles g dw $soil^{-1}$; upland soil, 2.0 – 2.7 µmoles g dw $soil^{-1}$; Figure S2). Also, NH_4^+ and NO_x concentrations in the inoculum was on average 8-10 folds higher in the paddy than upland soil, whereas PO_4^{3-} was comparable in both soils (Table 1). Higher inorganic N concentrations likely alleviated N limitation when coupled to high methane availability in the gamma-irradiated paddy soil, stimulating methanotrophic growth and methane uptake (< 12 days) when compared to the incubations containing the gamma-irradiated upland soil. However, we cannot exclude the availability of other N compounds (e.g., organic N) given the high total N in these soils (Table 1). Besides macronutrients, methanotrophic activity can be restricted by micronutrients (e.g., lanthanides, copper; Knapp et al., 2007; Semrau et al., 2010; 2018). Hence, we cannot completely exclude that differences in the micronutrient and trace element contents in the paddy and upland soils may have also affected methane oxidation rates. pH, and water content that may restrict methane and oxygen diffusion into the soil were similar during the incubation of both soils (Table 1; Supplementary Materials);

143 therefore, difference in methane uptake between the soils were likely caused by factors
144 other than these (Hiltbrunner et al., 2012; Shrestha et al., 2012). However, the water
145 potential which may affect water availability for microbial activity, potentially contributing to
146 shifts in the community composition, could be different in both soils (Harris, 1981).
147 Admittedly, not all abiotic factors potentially contributing to the difference in methane
148 uptake in both soils were determined. Nevertheless, it became evident that the soil abiotic
149 parameters more strongly affected the methanotrophic activity, resulting in significantly
150 higher methane uptake rates during the early stage (< 35 days) of recolonization under high
151 methane availability.
152
153 Microorganisms compete for nutrients as well as space, and occupy specific niches in the soil
154 according to their physiological requirements. These are examples for mechanisms shaping
155 the microbial activity and community composition (Little et al., 2008; Pan et al., 2014). Here,
156 the gamma-irradiated soils provide open niches, allowing rapid recolonization by the
157 inoculum-borne methanotrophs under high methane availability. The microcosms containing
158 native soils and their gamma-irradiated fractions (*PP* and *UU*) thus represent the optimum
159 native/gamma-irradiated soil combinations because the inoculum consists of
160 methanotrophs with established niche specialization (e.g., as a result of shared site history)
161 for the specific soil. Interestingly, although *PP* exhibited significantly higher methane uptake
162 than *PU* as anticipated, methane uptake in *UU* was significantly lower than *UP*, indicating
163 that conditions in the upland soil were not optimal and constrained methanotrophic activity;
164 the paddy soil likely possessed more suitable conditions (e.g., high inorganic N), favoring the
165 survival of methanotrophs. This suggests that the methanotrophic community composition,

166 although indigenous to the upland soil, plays a relatively less important role in determining
167 contemporary methane uptake rates than abiotic controls (Ho et al., 2016c).
168
169 *3.0 Methanotroph population dynamics; the emergence of the alphaproteobacterial*
170 *methanotrophs during recolonization.*
171
172 The total methanotroph abundance was at or below the detection limit of the qPCR assays
173 ($1.8 – 8.5 \times 10^5$ copy no. of target molecules g dw $soil^{-1}$) at the beginning of the incubations
174 and appreciably increased in all microcosms by approximately three to four orders of
175 magnitude during incubation (Figure 3), consistent with previous recolonization studies (Ho
176 et al., 2011; Pan et al., 2014). In particular, the significant increase ($p<0.01$) in all
177 methanotroph sub-groups (days 12-35; type Ia, four-fold; type Ib, seven-fold; type II, three-
178 fold) corroborated the higher total methane uptake in the *UP* incubations (Figures 2 and 3).
179 On the other hand, *PP* microcosms which exhibited similar total methane uptake to *UP*,
180 showed a significant increase ($p<0.01$) in type II methanotroph abundance during the same
181 time (Figure 3). Likewise, the abundance of type II methanotrophs significantly increased
182 ($p<0.01$) in the other incubations (from days 12-35 and days 12-19 in *PU* and *UU*,
183 respectively). Before being succeeded by the type II methanotrophs, type Ib methanotrophs
184 formed the majority in the paddy soil-inoculated microcosms (< 19 days; *PP* and *PU*), in
185 agreement with their general predominance in rice paddy environments (Lüke et al., 2013).
186 However, the high abundance of type Ib methanotrophs in *PU* is no consistent with the
187 relatively low methane uptake detected in this microcosm. This may be attributable to the
188 relatively more oligotrophic condition (e.g., lower inorganic N, NH_4^+, NO_3^-) or faster
189 transition to oligotrophic condition in the gamma-irradiated upland soil than in the gamma-

irradiated paddy soil incubations, resulting in lower cell-specific activity (Ho et al., 2011) not only in *PU*, but also in the *UU* microcosms. Although the methanotroph sub-groups were differentially affected during recolonization, the trajectory of the methanotroph succession was consistent across all microcosms, with the type II methanotrophs increasing in abundance and being generally more responsive than the type I during recolonization (> 12 days; Figure 3). This may reflect on the ecological characteristics of the community members in the native soil (see discussion below; Ho et al., 2017). Nevertheless, irrespective of the community members, it is likely that reduced competition coupled to high methane availability spurred recolonization.

The *pmoA* gene sequences, visualized as a principal component analysis (PCA; Figure 4) revealed a divergent community composition in the *PP*/*PU* and *UP*/*UU* incubations which could be largely separated along PC axis 2 (Figure 4). Over 90 % of the variation in the methanotrophic community composition could be explained by PC1 and PC2 (62.24% and 30.71% of the total variance, respectively). The predominant methanotrophs were represented by members of type Ia (*Methylobacter*), type Ib (Rice Paddy Clusters, RPCs), and type II (*Methylocystis*). The RPCs are putative methanotrophs closely related to *Methylocaldum* (Lüke et al., 2013; Shiau et al., 2019). Hence, the MiSeq sequencing enabled the identification of key methanotrophs within each sub-group. The PCA revealed a RPCs-dominated population in microcosms inoculated with native paddy soils (*PP* and *PU*), and *Methylobacter* dominated the community inoculated with native upland soils (*UP* and *UU*), with the *UU* incubation having a broader inventory of dominant methanotrophs comprising of *Methylocystis*, besides *Methylobacter*, reflecting on the dynamic shifts in the community composition. Hence, comparing the qPCR and *pmoA* gene sequencing analyses (Figures 3

214 and 4), the general trend in community dominance and succession was consistent in both
215 analyses.
216
217 When the same community was inoculated in different gamma-irradiated soils, similar
218 predominant communities developed, indicating that the initial community composition
219 plays a role in shaping the dynamics of the methanotrophic population, but not to an extent
220 that profoundly affects methane uptake. In particular, gammaproteobacterial
221 methanotrophs affiliated to *Methylobacter* and RPCs were predominant in the microcosms
222 inoculated with the upland and paddy soil, respectively, while type II methanotrophs related
223 to *Methylocystis* increased in abundance, more pronounced in the *UU* incubation (Figures 4
224 and S4). Previously, the potentially active community members when incubated near *in-situ*
225 methane concentrations in the paddy (~1%$_{v/v}$) and upland (30-40 ppm$_v$) soils were
226 predominantly comprised of type I and type II methanotrophs, respectively (Ho et al., 2013;
227 2019). Here, type I methanotrophs were initially dominant in the microcosms inoculated
228 with both soils. Considering that the gammaproteobacterial methanotrophs, particularly
229 members of type Ia (e.g., *Methylobacter*, *Methylosarcina*, *Methylomicrobium*,
230 *Methylomonas*), are thought to be more competitive under high or excess methane
231 availability, these methanotrophs may have been favored during incubation under high
232 (10%$_{v/v}$) methane concentrations (Krause et al., 2012; Reim et al., 2012; Ho et al., 2017 and
233 references therein). However, consistent in all incubations, type II methanotrophs
234 presumably comprised of *Methylocystis* significantly increased over time, and even
235 dominated the population after 19 days, despite of the prevalence of type I methanotrophs
236 (Figures 3 and 4). The emergence of *Methylocystis* during recolonization is not entirely
237 unexpected. Another alphaproteobacterial methanotroph (*Methylosinus*) showed

colonization potential in a soil and sediment, increasing in numerical abundance over time (< 3.5 months incubation; Ho et al., 2011; Pan et al., 2014). Likewise, in a synthetic community comprising of aerobic methanotrophs, only alphaproteobacterial ones (*Methylosinus* or *Methylocystis*) became dominant over time (Schnyder et al., 2018). The successional trajectory indicates that type II methanotrophs may become important for community functioning during late succession when conditions turned oligotrophic (e.g., after nutrient, including ammonium depletion; Ho et al., 2017).

Overall, results support our first supposition, indicating that methane oxidation is primarily governed by the soil physico-chemical properties, provided methane is available. The initial community composition influences the population dynamics of the methanotrophs without having pronounced effects on methane oxidation. Considering accumulating evidence indicating the relevance of biotic determinants in modulating methane oxidation (e.g., Ho et al., 2016a; Chang et al., 2018; Veraart et al., 2018), we further suggest that while soil edaphic properties modulate the methanotrophic activity at the pioneering stages of recolonization, biotic determinants (e.g., methanotrophic community structure, and interaction) may become relevant in established communities.

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All authors have seen and approved the final version of the manuscript.

Declarations of interest: none.

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Table 1: Selected physico-chemical parameters of the wetland and upland agricultural soils.

Soil (Coordinates)	Texture	pH*	Total C (µmoles C g dw soil^{-1})	Total N (µmoles N g dw soil^{-1})	Organic matter content (LOI %)	Total nutrient contents (µmoles g dw soil^{-1})			Vegetation (during sampling)	Reference
						NH_4^+	$NO_x^{\#}$	PO_4^{3-}		
Paddy soil (45^o 20′ N, 8^o 25′ E)	Calcareous clay	5.4	1158.3	92.9	4.0	1.0	0.3	$6.3x10^{-3}$	Rice (fallow)	Ho et al., 2015b
Upland soil (51^o 32′ N, 05^o 50′ E)	Gley podzol (sandy loam)	5.4	1850.0	92.9	4.7	0.1	$3.7x10^{-2}$	$9.5x10^{-3}$	Potato (fallow)	Ho et al., 2015a

Abbreviation: LOI, loss on ignition.

*pH determined in 1 M KCl (1:5, vol:vol).

$^{\#}$Total of NO_2^- and NO_3^-.

Figure captions

Figure 1: Experimental setup showing reciprocal inoculation of native soils in gamma-irradiated fractions of the soils. Three microcosms per time and amendment were established. The microcosms consisted of paddy soil + gamma-irradiated paddy soil (designated as '*PP*'), paddy soil + gamma-irradiated upland soil ('*PU*'), upland soil + gamma-irradiated paddy soil ('*UP*'), and upland soil + gamma-irradiated upland soil ('*UU*'). To confirm the sterility of the soil after gamma-irradiation, the soil (9.5 g) was saturated with autoclaved deionized water (0.45 ml g dw soil^{-1}) in a 120 ml bottle with an adjusted headspace methane concentration of 1 $\%_{v/v}$. The soil was considered free of viable methanotrophs when headspace methane remained unchanged over three weeks (Figure S1).

Figure 2: Methane uptake rate (A) and total methane consumed (B) during re-colonization (mean ± s.d.; *n*=3). Each soil microcosm consisted of 9.5 g gamma-irradiated soil and 0.5 g native soil in a Petri dish. The soil was saturated with autoclaved deionized water (0.45 ml g dw soil^{-1}) and homogenized before being incubated in a gas tight jar under 10 $\%_{v/v}$ methane in air in the dark at 27^{o}C. Headspace air in the jar was replenish every 2-3 days to ensure that methane was not limiting. At designated intervals (days 8, 12, 19, 26, and 35), individual microcosm was removed from the jar, and placed in a flux chamber to determine the methane uptake rate, measured over 5-6 hours (minimum of three time points) by linear regression (A), as described before (Ho et al., 2011). Negligible or no methane uptake was detected < 8 days in the *UP* and *UU* incubation hence, the first sampling was performed at day 12, allowing direct comparisons between treatments per time. Additionally, total

458 methane consumed for each treatment during the incubation (over 35 days) was
459 determined by integrating the area below the curve of methane uptake rates (B). Headspace
460 methane was measured using gas chromatography (GC) coupled to a thermal conductivity
461 and pulsed discharge helium ionization detector (7890B, Agilent Technologies, JAS GC
462 systems, Moers, Germany). In (B), letters indicate the level of significance (ANOVA; $p < 0.01$)
463 in the total methane consumed between treatments.
464
465 **Figure 3**: Response of the *pmoA* gene abundance of type Ia (A), type Ib (B), and type II (C)
466 methanotrophs during re-colonization. The qPCR was performed in duplicate for each DNA
467 extract (*n*=3), giving a total of six replicates per time, treatment, and assay. The lower case
468 letters indicate the level of significance (ANOVA; $p < 0.01$) between treatments per time. The
469 upper case letters indicate the level of significance (ANOVA; $p < 0.01$) between sampling
470 days per treatment. Values at the start of the incubation were at or below the detection
471 limits of the qPCR assays used. The lower detection limit of the qPCR assays is indicated by
472 the dashed line ($1.8 – 8.5 \times 10^5$ copy no. of target molecules g dw soil^{-1}).
473
474 **Figure 4**: Principal component analysis (PCA) showing the response of the methanotrophic
475 community composition during recolonization. The composition of the methanotrophic
476 community was derived from sequencing of the *pmoA* gene performed for each DNA extract
477 (*n*=3) per time and treatment. The PCA was performed in the R statistics software
478 environment (R core Team, 2014) using the function 'prcomp'. Visualization of the PCA was
479 performed using the 'ggfortify' package. Rarefaction curves generated for each sample
480 showed a good coverage of the *pmoA* gene diversity (Figure S3). The affiliation and
481 distribution of the *pmoA* gene sequences are given in Figure S4. The vectors indicate

predominant methanotrophic genera/group. Abbreviations: RPC, rice paddy cluster (type Ib-related methanotroph).

Figure 1
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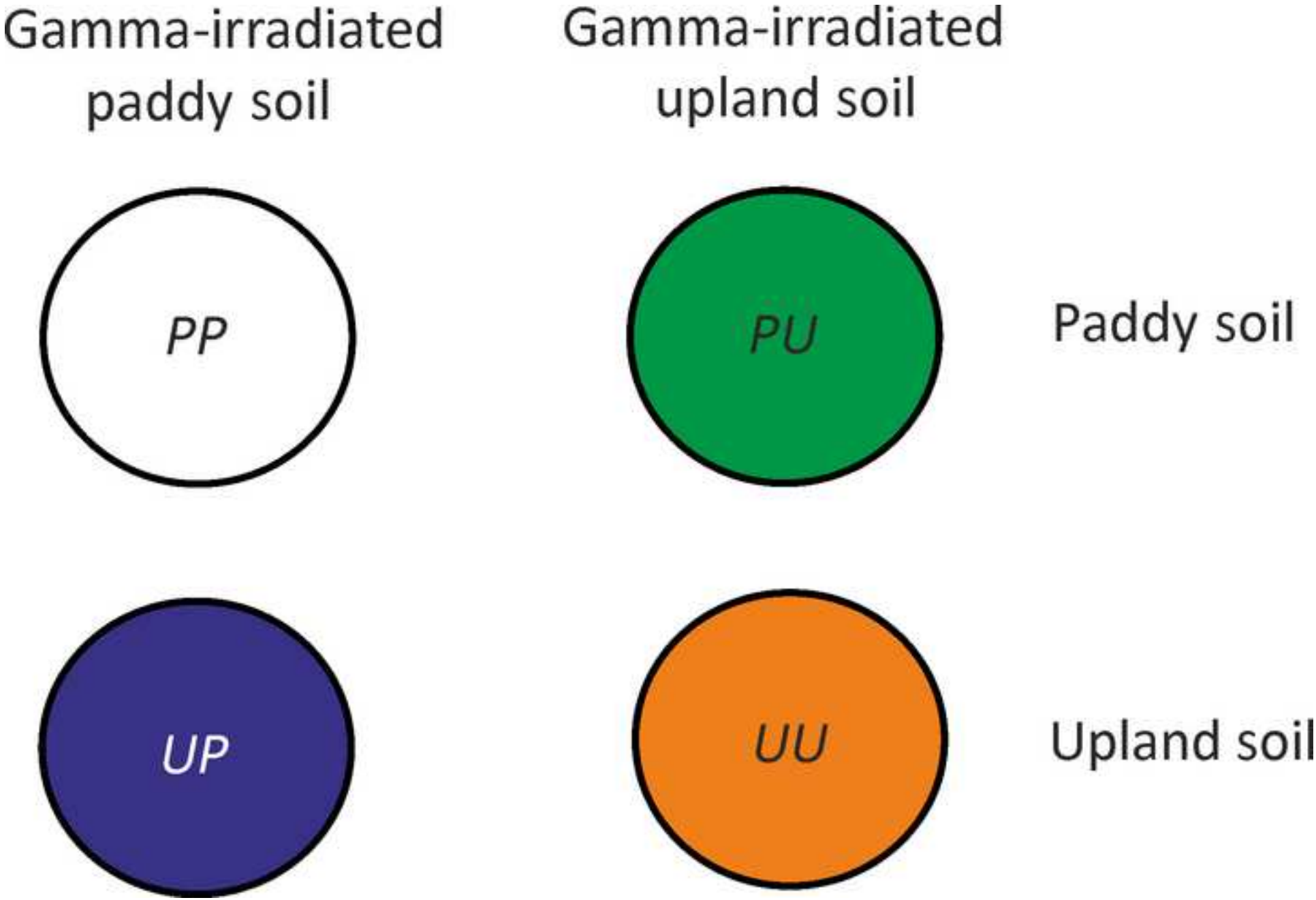

Figure 2
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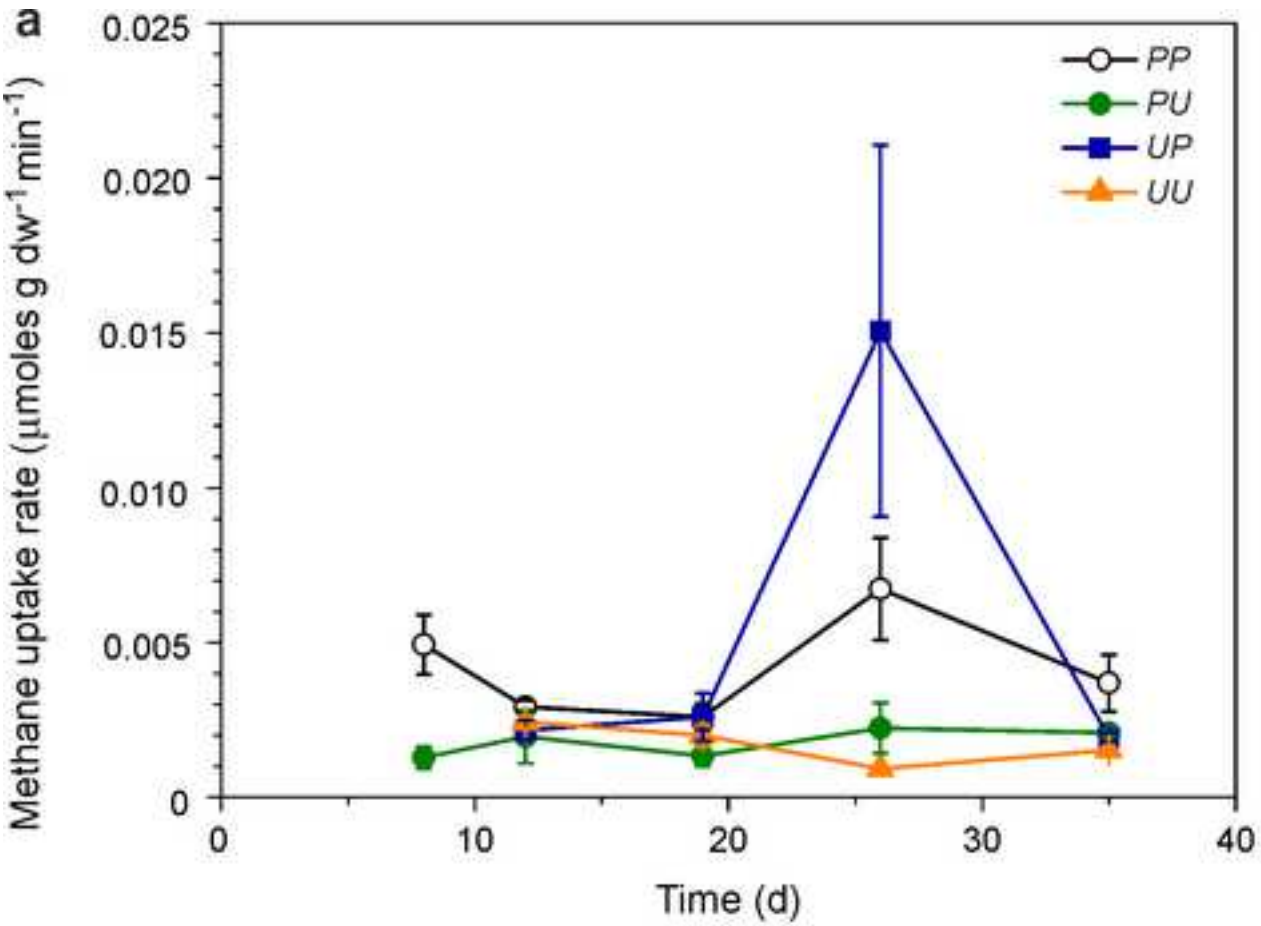

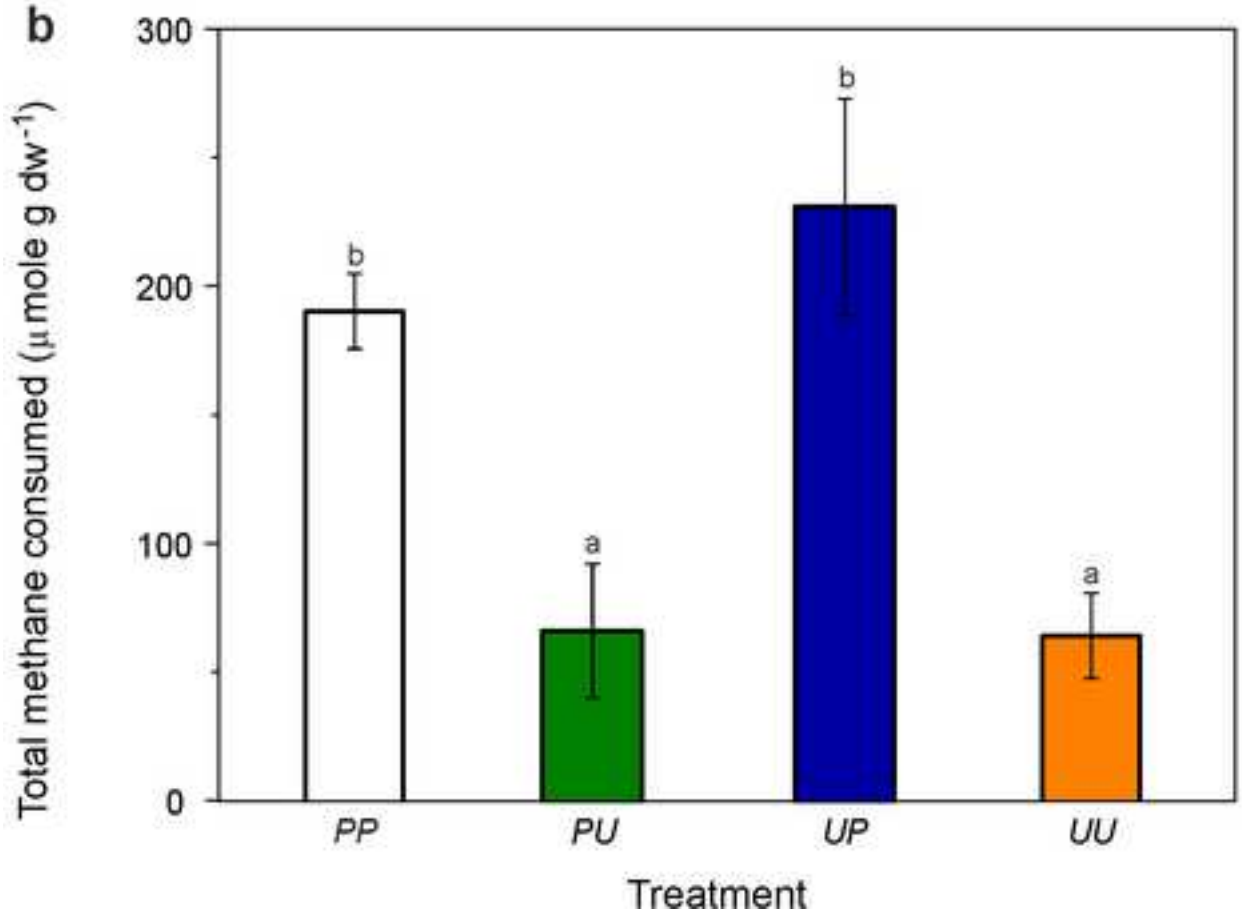

Figure 3

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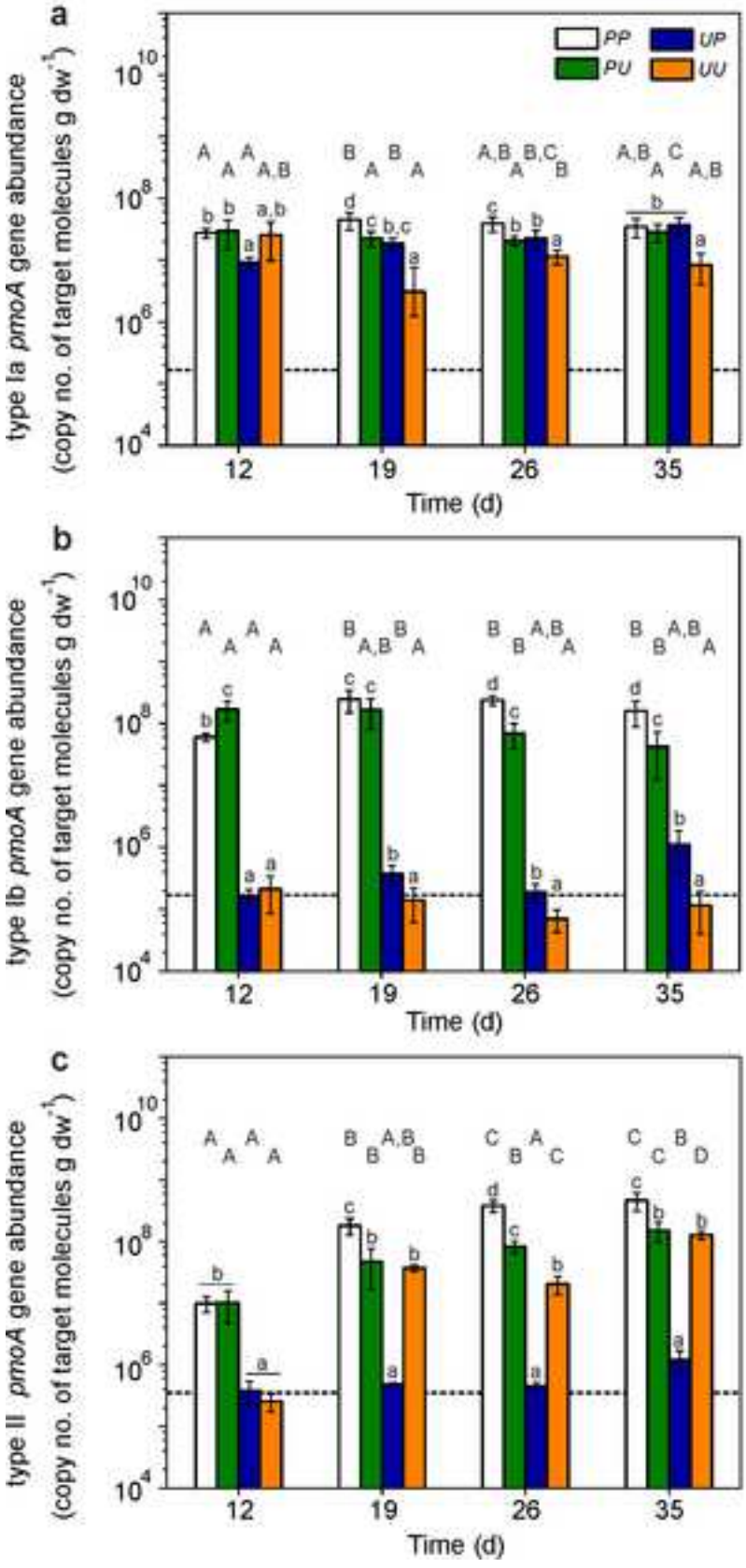

Figure 4

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