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Methanotrophic activity in slurry surface crusts as influenced by CH₄, O₂, and inorganic N

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Abbreviations

MOB: Methane oxidizing bacteria; PLFA: Phospholipid fatty acid; FAME: Fatty acid methyl esters

Keywords

Methane oxidation; Inorganic nitrogen; Microsensor; PLFA stable-isotope probing; Methane oxidizing bacteria.

Core Ideas

- Oxygen penetration into surface crusts is shallow.
- Nitrous oxide accumulates at oxic-anoxic interfaces in surface crusts.
- Oxygen availability is important to high-concentration methane oxidation.
- Microbial methane oxidation is affected by interactions of inorganic N and oxygen.
- Activity of Type I methanotrophs dominates under high methane concentration.

1 **Abstract**

2 Livestock slurry is a major source of atmospheric CH₄, but surface crusts harboring methane oxidizing
3 bacteria (MOB) could mediate against CH₄ emissions. This study examined conditions for methane oxidation
4 by *in-situ* measurements of O₂ and N₂O, as a proxy for inorganic N transformations, in intact crusts using
5 microsensors. This was combined with laboratory incubations of crust material to investigate effects of O₂,
6 CH₄, and inorganic N on methane oxidation, using ¹³CH₄ to trace C incorporation into lipids of MOB. Oxygen
7 penetration into the crust was 2–14 mm, confining the potential for aerobic methane oxidation to a shallow
8 layer. Nitrous oxide accumulated within or below the zone of O₂ depletion. With 10² ppmv CH₄ there was no
9 O₂ limitation on methane oxidation at O₂ concentrations as low as 2%, whereas methane oxidation at 10⁴
10 ppmv CH₄ was reduced at ≤ 5% O₂. As hypothesized, methane oxidation was in general inhibited by inorganic
11 N, especially NO₂⁻, and there was an interaction between N inhibition and O₂ limitation at 10² ppmv CH₄, as
12 indicated by consistently stronger inhibition of methane oxidation by NH₄⁺ and NO₃⁻ at 3% compared to 20%
13 O₂. Recovery of ¹³C in phospholipid fatty acids suggested that both Type I and Type II MOB were active, with
14 Type I dominating high-concentration methane oxidation. Given the structural heterogeneity of crusts,
15 methane oxidation activity likely varies spatially as constrained by the combined effects of CH₄, O₂, and
16 inorganic N availability in microsites.

17 **Introduction**

18 In regions with intensive livestock production such as Western Europe and North America, up to 40% of
19 livestock CH₄ emissions may be related to manure management (Francesco et al., 2013). In most cases, CH₄
20 capture and/or biofiltration is neither technically feasible nor economical (Melse and van der Werf, 2005),
21 and more cost-effective alternatives must be considered. When manure is stored as liquid slurry, a dense
22 floating crust is often formed either naturally from dry matter in the slurry, or by facilitation of admixing
23 with chopped straw (Hansen et al., 2009). Studies have demonstrated a potential for aerobic methane
24 oxidation in such surface crusts (Petersen and Ambus, 2006; Petersen et al., 2005), where diverse
25 communities of methane-oxidizing bacteria (MOB) were also documented (Duan et al., 2014). These findings

26 suggest that surface crusts could act as a low-cost filter for manure-derived CH₄, but the physical, chemical,
27 and biological regulation of methane oxidation inside crusts is largely unknown.

28 Methane oxidation potential depends on CH₄ and O₂ availability, which are highly variable due to the
29 heterogeneous structure of the crust. Since surface crusts overlie liquid manure with a high methanogenic
30 potential, they are typically high-CH₄ environments with concentrations far above the atmospheric level. We
31 have observed up to 200 ppmv CH₄ in the stagnant atmosphere immediately above the surface crust (Y.F.
32 Duan, unpublished data), and headspace CH₄ concentrations of 10² and 10⁴ ppmv were used previously to
33 simulate this range of CH₄ availability for MOB in laboratory incubations (Duan et al., 2013). Due to the often
34 loose structure of the crust, sectioning for extraction and determination of *in situ* CH₄ availability by
35 sampling are impractical. While a CH₄ biosensor has been described (Damgaard and Revsbech, 1997), it is
36 not commercially available and has not been tested in heterogeneous environments such as surface crusts.
37 In contrast to CH₄, the distribution of O₂ in surface crusts can be readily determined using a microsensor
38 (Revsbech, 2005), as demonstrated by Hansen et al. (2009) and Nielsen et al. (2010) who investigated O₂
39 penetration into various slurry crusts.

40 Inhibition of methane oxidation by inorganic N is known from many environments (Bosse et al., 1993;
41 Dunfield and Knowles, 1995; Wang and Ineson, 2003), as well as in surface crusts (Duan et al., 2013), but
42 stimulation or no effect can also occur (Liu and Greaver, 2009). Livestock slurry and surface crusts are highly
43 enriched in inorganic N (Table 1): the slurry phase may contain up to 200 mM ammoniacal N (NH₃/NH₄⁺)
44 (Nielsen et al., 2010; Sommer et al., 2007), while up to 35 mM NO₃⁻ (Hansen et al., 2009) and 98 mM NO₂⁻
45 (Nielsen et al., 2010) have been found at the oxic-anoxic interface in surface crusts. These concentration
46 ranges (Table 1) represent a high variability of inorganic N at microsites within surface crusts due to
47 fluctuations in water content as a result of precipitation and insolation (Nielsen et al., 2010). Due to this
48 heterogeneity, bulk N concentrations are insufficient to characterize N distribution and thus potential
49 interference with methane oxidation in microsites. While specialized sensors are available to determine
50 micro-scale concentrations of NH₄⁺, NO₂⁻, and NO₃⁻ in aqueous environments (De Beer et al., 1997; De Beer

51 et al., 1991; Larsen et al., 1996), they are difficult to apply in unsaturated matrices such as surface crusts,
52 where contact to liquid phase may be intermittent. Instead, the accumulation of N₂O in the crust was used in
53 this study as a proxy for the distribution of N transformations and thus presence of inorganic N (Larsen et al.,
54 1996), as both nitrification and denitrification can produce N₂O under sub-oxic conditions (Bollmann and
55 Conrad, 1998).

56 The effects of environmental factors on methane oxidation in surface crusts will ultimately depend on the
57 MOB present (Bodelier, 2011; Hu and Lu, 2015). Aerobic MOB have been conventionally categorized into
58 Type I and Type II based on phylogenetic and functional traits, including the presence of signature 16-carbon
59 (C₁₆) or 18-carbon (C₁₈) phospholipid fatty acids (PLFAs) (Hanson and Hanson, 1996). Type I MOB have been
60 reported to thrive in N-sufficient, high-O₂ and low-CH₄ environments, whereas Type II MOB seem to favor
61 the opposite (Amaral et al., 1995; Amaral and Knowles, 1995; Graham et al., 1993). It is still unclear to what
62 extent CH₄, O₂, and inorganic N conditions will select one type of MOB over another in surface crusts, but
63 previous studies found that Type I MOB are more abundant and diverse than Type II MOB in this
64 environment (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2013). In recent years, novel methane
65 oxidation pathways, such as NO₂⁻ dependent anaerobic methane oxidation (Ettwig et al., 2010; Welte et al.,
66 2016), as well as aerobic methane oxidation coupled with partial denitrification (Kits et al., 2015a; Kits et al.,
67 2015b), have also been described, but the importance of these processes and the presence of relevant
68 microorganisms in surface crusts remain unclear.

69 A main objective of this study was to examine the potential for microbial methane oxidation under realistic
70 storage conditions by characterizing *in situ* distributions of O₂ and inorganic N transformations in a cattle
71 slurry surface crust using microsensors. Effects and interactions of O₂ and inorganic N species with respect to
72 methane oxidation could not be quantified *in situ* where microbial activities occur in micro-sites, and instead
73 this was investigated under controlled laboratory conditions. Here, ¹³CH₄ was used as substrate, allowing ¹³C
74 stable isotope probing of PLFAs to study the involvement of Type I and Type II MOB in methane oxidation in
75 surface crusts. Based on previous results (Duan et al., 2014; Duan et al., 2013) we hypothesized that

76 methane oxidation activity in surface crusts would be determined by both CH₄ and O₂ availability, and
77 inhibited by inorganic N, and that Type I MOB would be primarily responsible for methane oxidation.

78 **Materials and Methods**

79 ***Microsensor Measurement of O₂ and N₂O Distribution***

80 Dairy cattle slurry was collected from a full-scale manure storage facility in May, 2012, and transferred to
81 two tanks at a pilot-scale storage system (Petersen et al., 2009) at Aarhus University (Foulum, Denmark). The
82 slurry was stored for six weeks prior to the measurement in June, by which time a 5–6 cm thick surface crust
83 with a stable structure had developed on top of the slurry. The development of surface crust reflected
84 typical storage conditions, where a new crust is formed following the mixing of slurry in spring for field
85 application.

86 Oxygen and N₂O concentration profiles in the surface crust were determined using, respectively, an O₂ and a
87 N₂O microsensor with a tip diameter of 0.5 mm (both produced by Unisense, Aarhus, Denmark). Both
88 microsensors were calibrated according to manufacturer's instructions. Detection limits for O₂ and N₂O were
89 0.3 μmol L⁻¹ and 0.1 μmol L⁻¹, respectively.

90 A custom-made mounting system was used to place the microsensors over the surface crust (Supplemental
91 Fig. S1). Oxygen profiles were recorded at 20-cm intervals from 20 to 180 cm along the 200-cm diameter of
92 the storage tank. During measurement, the microsensor was introduced stepwise into the crust at 0.5 mm
93 increments to a maximum depth of 30 mm using a motorized micromanipulator (Unisense). At each depth,
94 the microsensor was stationary for 3 s to allow gas equilibration, and then the O₂ concentration was
95 determined as the average of a 3 s reading. The signal was amplified by a multimeter (Unisense), and
96 registered by the SensorTrace PRO v3.0 software (Unisense). Nitrous oxide profiles were determined at the
97 same locations as O₂ profiles, but with a +2 mm offset to avoid any disturbance to the crust caused by the O₂
98 microsensor. The N₂O profiles were recorded with the same procedure as O₂ profiles, and initially over the

99 same depth. However, due to incidences of significant N₂O accumulation at 30 mm depth, the maximum
100 depth was extended to 60 mm starting from the 5th profile (at 100 cm distance from the edge).

101 Due to the uneven surface of the crust, a fixed depth cannot accurately define the crust-air interface. For O₂,
102 an abrupt decrease from atmospheric concentration defined the crust-air interface. For N₂O, which was
103 produced inside the crust and diffused towards the atmosphere, the crust-air interface was defined as the
104 depth where N₂O concentration dropped below the detection limit. Using these two criteria, the O₂ and N₂O
105 concentration profiles were aligned.

106 ***Methane Oxidation in Response to O₂ and N Amendments***

107 Surface crust was collected from a full-scale storage tank at the biogas plant of Aarhus University in March,
108 2012. The 10-cm surface crust had developed on slurry co-digested with maize silage. Homogenized crust
109 samples were stored in closed plastic containers at 2°C until used for experiments within four weeks. Duan
110 et al. (2013) showed that MOB can survive and recover activity under these storage conditions for at least
111 three months.

112 Methane oxidation rates were determined by incubating 3-g crust samples in liquid media under a controlled
113 atmosphere. To reduce background N, crusts were washed three times by vortexing with 20 mL deionized
114 water followed by centrifugation at 10,000 × *g* for 10 min. The washing did not remove all NH₄⁺, but the
115 residual NH₄⁺ was negligible compared to the received NH₄⁺ amendment (Duan et al., 2013). Washed crust
116 materials were transferred to 125 mL serum bottles, and resuspended in 20 mL basal salt (BS) medium
117 prepared according to Whittenbury et al. (1970), but excluding N salts. The BS medium was dispensed using
118 a customized system designed to remove dissolved O₂ and maintain anoxia during sample preparation
119 (Supplemental Fig. S2).

120 Various treatments with combinations of different CH₄, O₂, and inorganic N were prepared (Table 2). For N
121 amendments, pre-made solutions of (NH₄)₂SO₄, KNO₃, or KNO₂ were injected into the serum bottles to

122 achieve the desired N concentrations. The bottles were purged using a vacuum pump and refilled with
123 helium, and this step was repeated three times to exhaust residual O₂. Then, air and ¹³C-labeled CH₄ (99
124 atom% ¹³C, ISOTECH, Miamisburg, OH, USA) were injected to achieve the desired headspace gas
125 concentrations. To meet the requirement for CO₂ by some MOB, 4 mL pure CO₂ was also added (Acha et al.,
126 2002). After gas injection, the bottles were mounted on a rotary shaker at 150 rpm for 30 min to allow for
127 liquid-gas equilibration. Headspace O₂ concentrations were verified using an Agilent 3000A MicroGC
128 (Hørsholm, Denmark) as described by (Petersen et al., 2009). For treatments with 0% O₂, residual headspace
129 O₂ concentration was undetectable (< 10–20 ppmv). The bottles were then incubated on a rotary shaker at
130 200 rpm at ca. 21°C; Duan et al. (2013) had shown that there is no gas diffusion limitation under these
131 incubation conditions. Headspace CH₄ concentrations were measured after 0, 2, 4, 6, 8, 24, 48, and 72 h
132 using a Shimadzu 14B GC as described by Duan et al. (2013).

133 In each batch of assays, a control with crust material in N-free BS medium at atmospheric O₂ concentration
134 was included to check for batch-to-batch variations in crust MOB activity, and a blank control without crust
135 material to correct for loss of pressure during repeated gas samplings.

136 First-order rate constants for the first 8 h of incubation and relative activities were calculated according to
137 Duan et al. (2013). First-order rate constants were compared between treatments using R v3.2.2 (R Core
138 Team, 2015). For each CH₄ concentration, effects of O₂ and inorganic N, and their interaction, were analyzed
139 by a two-way ANOVA. Differences between treatments were determined by Duncan's post-hoc multiple
140 comparison test.

141 ***PLFA Extraction and GC-c-IRMS Analysis***

142 After incubation with ¹³C-labeled CH₄, selected crust samples were processed for PLFA analysis (Table 2).
143 Prior to lipid extraction it was necessary to reduce the organic load since otherwise the humic material
144 would bind the chloroform phase and prevent isolation of lipid-soluble compounds. Crust samples were
145 vortexed and centrifuged for 10 min at 3000 × g to extract microbial cells. The supernatant was filtered

146 through 0.2 μm chloroform-soluble polycarbonate filters, and the material retained on the filter was used
147 for lipid extraction. Hence, lipid results refer to the fraction of extractable low particle size material only.
148 Polar lipid fatty acid methyl esters (FAMEs) from each filter were prepared as previously described by
149 Petersen et al. (2002).

150 FAMEs were analyzed using a HP6890 GC (Agilent, Santa Clara, CA, USA) coupled via a GC combustion
151 interface (Thermo Scientific, Bremen, Germany) in continuous flow mode to a Finnigan Delta^{PLUS} isotope
152 ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The oxidation reactor on the interface was
153 maintained at 940 °C, the reduction reactor at 650 °C. Samples (1 μL) were injected at 240 °C in splitless
154 mode. The column temperature was held at 50 °C for 2 min, then increased at 15 °C min^{-1} to 100 °C,
155 subsequently at 2 °C min^{-1} to 220 °C, and finally at 15 °C min^{-1} to 240 °C, where the final temperature was
156 held for 5 min. Separated compounds were measured against a CO_2 reference gas calibrated with reference
157 to Vienna PeeDee belemnite. PLFAs were identified by relative retention time comparing samples against a
158 FAME standard mix (Supelco 37 component FAME mix, 47885-U, Sigma Aldrich). All $\delta^{13}\text{C}$ values were
159 corrected for the methanol C added during methanolysis:

160

$$\delta^{13}\text{C}_{\text{FAME}} = \frac{(N_{\text{FA}} + 1)\delta^{13}\text{C}_{\text{FAME}} - \delta^{13}\text{C}_{\text{MeOH}}}{N_{\text{FA}}}$$

161 where N_{FA} refers to the number of carbon atoms of the fatty acid component, $\delta^{13}\text{C}_{\text{FAME}}$ is the observed $\delta^{13}\text{C}$
162 value of the FAME, and $\delta^{13}\text{C}_{\text{MeOH}}$ is the $\delta^{13}\text{C}$ value of the methanol used for methanolysis ($-37.7\text{‰} \pm 3.2\text{‰}$).
163 The $\delta^{13}\text{C}$ isotope ratios were converted to atom%, and atom% excess was then calculated by subtraction of
164 an unlabeled control. The incorporation of $^{13}\text{CH}_4$ into membrane PLFAs ($n_{13\text{C}}$, nmol) was calculated as $n_{13\text{C}} =$
165 $(\text{PA}_{\text{FAME}} / \text{PA}_{\text{ISME}}) \times n_{\text{ISME}} \times (\text{atom}\% \text{ } ^{13}\text{C} \text{ excess})$, where PA_{FAME} and PA_{ISME} are peak areas of the FAME and
166 internal standard *Me19:0*, respectively, and n_{ISME} (nmol C) is the concentration of the internal standard fatty
167 acid. The lower limit of identified peaks corresponded to 0.1 ng $^{13}\text{C} \text{ g}^{-1}$ crust material.

168 In view of the uncertain recovery of microbial cells following centrifugation and filtration, statistical testing
169 of treatment effects was not performed, and results will only be presented as means \pm standard errors.

170 **Results**

171 ***Oxygen and N₂O Distribution in Natural Surface Crusts***

172 Surface crusts from the two storage tanks showed a high spatial variability in shape and penetration depth of
173 individual O₂ and N₂O profiles, yet the distribution patterns were qualitatively similar between the two
174 tanks. Thus, results presented here are from one of the storage tanks only (Fig. 1).

175 Oxygen penetration depth varied from 2 to 14 mm, with either a steep or more gradual decline in
176 concentration. Irregularities such as a secondary increase following the initial decline were also observed
177 (e.g., at 60 cm).

178 Nitrous oxide profiles showed peak concentrations at 5–25 mm depth below the crust-air interface. Some
179 N₂O profiles consisted of more than one zone of N₂O accumulation (e.g., at 60, 100, and 160 cm). Maximum
180 N₂O accumulation often coincided with sub-oxic or anoxic zones indicated by O₂ profiles. In a few cases (e.g.,
181 at 60 and 180 cm), N₂O production took place where O₂ availability was relatively high.

182 ***Response of CH₄ Oxidation to O₂ Concentrations***

183 Figure 2 shows the changes in headspace CH₄ concentrations during the 72-h incubation study with different
184 initial O₂ levels and two initial CH₄ levels. For both 10² and 10⁴ ppmv CH₄, consistent methane consumption
185 throughout the incubation was observed only at 20% initial O₂, with a > 90% decline in headspace CH₄
186 concentrations after 72 h. In treatments with \leq 5% initial O₂, CH₄ consumption was generally observed within
187 the first 24 h, followed by net CH₄ accumulation. At 0% initial O₂, net CH₄ production was observed
188 throughout the incubation.

189 The strength of methane oxidation activity was expressed as first-order rate constants during the first 8 h of
190 incubation (Fig. 3). At 10^2 ppmv initial CH_4 there were no significant differences in first-order rate constants
191 at O_2 levels from 20% down to 2%, but at 1% O_2 the rate was significantly impaired. At 10^4 ppmv initial CH_4 ,
192 methane oxidation rates were significantly reduced at lower O_2 levels, though not significantly different
193 between 2% and 1% O_2 .

194 ***Response of CH_4 Oxidation to Interactions between O_2 and Inorganic N***

195 When samples with manipulated O_2 and CH_4 concentrations were amended with inorganic N, complex
196 patterns in microbial methane oxidation were observed. For each of the two initial CH_4 concentrations, two-
197 way ANOVA showed significant effects of O_2 concentrations and N amendments, as well as their interaction,
198 on methane oxidation rates.

199 At 10^2 ppmv CH_4 , first-order rate constants in all N-amended samples were lower than in the N-free control
200 (Table 3). The inhibition was strengthened at increasing concentrations of both NH_4^+ and NO_3^- . Samples
201 treated with NO_3^- consistently showed less inhibition than treatments receiving other N salts, whereas NO_2^-
202 was a potent inhibitor as indicated by similar inhibitions with 1 mM NO_2^- and 50 mM NH_4^+ . At 3% O_2 ,
203 inhibition by individual N species and concentrations was slightly stronger than at 20% O_2 , but the difference
204 was not always statistically significant.

205 At 10^4 ppmv CH_4 the order of inhibition by different N species was similar to that at 10^2 ppmv CH_4 , with NO_2^-
206 as the strongest inhibitor (Table 3). Yet, several NO_3^- treatments caused a weak stimulation rather than
207 inhibition as compared to the N-free control. Generally, the N amendments inhibited methane oxidation at
208 20% O_2 , but not at 3% O_2 , where low rates were already observed in the N-free control. One exception,
209 though, was the inhibition caused by the treatment with 50 mM NH_4^+ .

210 ***¹³C Incorporation into C₁₆ and C₁₈ PLFAs***

211 The yields of PLFA varied considerably between samples, and ¹³C incorporation was below the detection limit
212 for several PLFAs, precluding a detailed quantitative analysis. Instead of absolute PLFA concentrations, an
213 index based on peak area was calculated (Fig. 4, a1–a4). Recovery of ¹³C PLFAs was consistently low in
214 incubations with 10² ppmv CH₄ (Fig. 4, a1). At 10⁴ ppmv CH₄ there was an 8–9 times higher ¹³C recovery in
215 PLFAs at 20% and 3% O₂ than at 1% O₂ (Fig. 4, a2). While NH₄⁺ and NO₃⁻ considerably reduced ¹³C recovery,
216 NO₂⁻ caused no or only moderate inhibition of ¹³C recovery (Fig. 4, a3 and a4). The total recovery of ¹³C was
217 5–15 times higher with NO₂⁻ amendment than with the other two N species.

218 Two ¹³C-labeled PLFA clusters, C₁₆ and C₁₈, were defined in accordance with the predominance of these
219 PLFAs in Type I and Type II MOB, respectively (Bodelier et al., 2009). The C₁₆ cluster included peaks identified
220 as 16:0, 16:1ω6, 16:1ω7, and 16:1ω8, whereas the C₁₈ cluster included 18:0, 18:1ω7, 18:1ω9, and probably
221 also small peaks of 18:1ω8. Also, 16:1ω6 probably co-eluted with 10Me16:0, and 16:1ω8 with i17:0, but this
222 did not influence the calculated ¹³C incorporation for the cluster.

223 Due to the low recovery of total ¹³C PLFA (Fig. 4, a1) and low percentage (3–9%) of ¹³C in C₁₆ and C₁₈ clusters
224 at 10² ppmv CH₄ (Fig. 4, b1), no detailed interpretation of these results was possible. At 10⁴ ppmv CH₄ the
225 recovery of ¹³C in the C₁₆ and C₁₈ clusters together accounted for an average of 52% of ¹³C recovered in
226 PLFAs. A higher percentage of ¹³C was always recovered in the C₁₆ than in the C₁₈ cluster, and the percentage
227 of the C₁₆ cluster was particularly high in treatments with NO₂⁻ amendment (Fig. 4, b3 and b4).

228 **Discussion**

229 Due to the heterogenous nature of surface crusts, microbial activities within this environment are controlled
230 by physical and chemical properties of individual microsites rather than overall bulk properties. However,
231 detailed analysis of surface crusts is challenged by the often loose and fibrous structure of the material, and
232 this was also the case with the straw-containing cattle slurry crust used in this study. Therefore, we chose to

233 characterize O₂ and inorganic N distributions *in situ* by microsensors, while regulation of microbial activities
234 were investigated by controlled laboratory incubations. For logistic reasons, the *in situ* gas measurements
235 and laboratory incubations were performed using different surface crusts. However, previous studies have
236 shown that crusts of different origin are qualitatively similar with respect to, e.g., depth of O₂ penetration
237 and the presence of MOB (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2010; Nielsen et al., 2013),
238 and results from the two parts are therefore analyzed and discussed together.

239 ***Effects of O₂ Limitation and N Amendments***

240 Methane oxidation kinetics are complex as the reaction involves two substrates, CH₄ and O₂ (Cai and Yan,
241 1999). We were not able to monitor O₂ concentrations during incubation, but instead calculated O₂
242 consumption by MOB based on the amounts of CH₄ consumed, the stoichiometry of methane oxidation
243 (Urmann et al., 2007), and the diffusion coefficients of CH₄ and O₂ in water (Broecker and Peng, 1974)
244 (Supplemental Table S1). These calculations suggested that there was no diffusional limitation of O₂ for
245 methane oxidation, and that the amounts of O₂ used for methane oxidation were < 10% of the available O₂
246 even at 1% initial O₂. Thus, depletion of O₂ during incubation must have been mainly due to aerobic
247 processes other than methane oxidation, i.e., any O₂ limitation for MOB activity reflected competition for O₂
248 against other aerobes. Headspace CH₄ concentrations decreased exponentially during the first 8 h, indicating
249 that the rates of CH₄ uptake depended mainly on CH₄ availability. Therefore, the reaction was approximated
250 by first-order reaction kinetics, which have also been used previously in studies of microbial methane
251 oxidation (De Visscher et al., 1999; King and Schnell, 1994; King and Schnell, 1998; Petersen and Ambus,
252 2006). The apparent first-order kinetics suggest that the rate of CH₄ uptake, and thus demand for O₂, was
253 proportional to the CH₄ concentration, which explains our observation that methane oxidation became more
254 affected by O₂ limitation at 10⁴ ppmv than at 10² ppmv CH₄ (Fig. 3).

255 The neutral to alkaline pH of slurry (Nielsen et al., 2010; Petersen and Ambus, 2006) suggests the presence
256 of free ammonia (NH₃), which is a competitive inhibitor for methane oxidation (Carlsen et al., 1991; Gullledge

257 and Schimel, 1998). In both the present (Table 3) and a previous study (Duan et al., 2013), inhibition by
258 ammonia was less at 10^4 compared to 10^2 ppmv CH_4 , suggesting that competitive inhibition is important for
259 effects of ammonia. The observations that NO_2^- was a more potent inhibitor of methane oxidation than NO_3^-
260 was also consistent with the report by Duan et al. (2013), where the concentration of NO_3^- resulting in 50%
261 inhibition was 100-fold higher than that of NO_2^- . It is likely that these N species both inhibit methane
262 oxidation *via* nitrite toxicity (Stein and Klotz, 2011).

263 We further tested interactions of N inhibition with O_2 availability by comparing relative activities. If there
264 were an interaction between O_2 and inorganic N, a given N amendment would result in different degree of
265 inhibition at 20% and 3% O_2 concentrations. At 10^2 ppmv initial CH_4 , 10 mM, but not 50 mM, NH_4^+ or NO_3^-
266 amendments caused a stronger inhibition at 3% than at 20% O_2 (Table 3), confirming an interaction between
267 N inhibition and O_2 limitation at low N concentrations. At 10^4 ppmv CH_4 , the fact that N amendments
268 generally inhibited methane oxidation at 20% O_2 but not further at 3% O_2 suggested that high-concentration
269 methane oxidation is more sensitive to O_2 limitation rather than to N inhibition.

270 Generally, as an essential substrate for aerobic methane oxidation, O_2 has a direct and immediate effect on
271 aerobic methanotrophic activity. On the other hand, the mechanism of N inhibition on methane oxidation is
272 much more complex and may include immediate toxicity to cell growth and enzyme synthesis, as well as
273 delayed influence on microbial community composition (Bodelier and Laanbroek, 2004). The mechanisms
274 behind interactions between controlling factors in surface crusts could not be explained with the data
275 presented here, and more research is needed to further elucidate this matter.

276 ***^{13}C PLFA Signatures for MOB***

277 Type I and Type II MOB produce unique membrane PLFAs, 16:1 ω 8 and 18:1 ω 8, respectively (Bodelier et al.,
278 2009; Hanson and Hanson, 1996). These signature PLFAs are not always present, or present only in low
279 amounts. However, there is also a general predominance of C_{16} and C_{18} PLFAs among Type I and Type II
280 MOB, respectively (Bodelier et al., 2009), and this has been used to evaluate sources of methane oxidation

281 by stable isotope probing (Qiu et al., 2008). In the present study, both types of MOB were active, with Type I
282 MOB dominating the methanotrophic activity especially where CH₄ availability was high (Fig. 4, b1 and b2).
283 Molecular analyses of microflora in other crust materials likewise suggested that Type I MOB dominated the
284 methanotrophic community in terms of both diversity and abundance (Duan et al., 2014).

285 Interestingly, the incorporation of ¹³C into PLFAs was high in surface crusts incubated with NO₂⁻ as compared
286 to those with other N species (Fig. 4, a3 and a4), especially considering that crusts incubated with NO₃⁻ had
287 over two-fold higher ¹³CH₄ uptake than NO₂⁻ treatments (data not shown). Roslev et al. (1997) reported that
288 NH₄⁺ decreased C conversion efficiency and increased respiration of C assimilated by MOB, which is
289 consistent with the low ¹³C recovery from NH₄⁺ treatments in this study. However, the mechanism by which
290 NO₃⁻ and NO₂⁻ could interfere with C assimilation for MOB remains unclear. Alternative pathways for CH₄
291 uptake may have contributed to the particularly high ¹³C assimilation in NO₂⁻ amended crusts. The
292 microorganism *Candidatus Methyloirabilis oxyfera* is able to couple anaerobic methane oxidation with
293 nitrite reduction (Ettwig et al., 2010), and may be widespread in natural environments (Ettwig et al., 2009;
294 Wang et al., 2012; Zhu et al., 2012). We recovered 10MeC_{16:0} in the present study, which is characteristic of
295 the lipid profile of *M. oxyfera* (Kool et al., 2012). Still, more concrete evidence, such as the recovery of
296 specific gene markers of *M. oxyfera* (Luesken et al., 2011), is needed to confirm the presence of *M. oxyfera*
297 in surface crusts. More recently, gammaproteobacterial (Type I) methanotrophs *Methylomonas denitrificans*
298 and *Methylomicrobium album* have been reported to be able to oxidize methane under hypoxia using
299 oxidized nitrogen as electron acceptor (Kits et al., 2015a; Kits et al., 2015b). The involvement of such a
300 process would be consistent with the high proportion of C₁₆ PLFAs recovered from NO₃⁻ and NO₂⁻
301 treatments, and the genera *Methylomonas* and *Methylomicrobium* are widespread (Knief, 2015) and were
302 indeed present in surface crusts (Duan et al., 2014). However, there was no direct evidence in the present
303 study to evaluate the presence of these specific strains.

304 ***In-situ O₂ and N₂O Distribution and Implications for CH₄ Oxidation***

305 Oxygen distribution in surface crusts vary over short distances, as shown in this and other studies (Hansen et
306 al., 2009; Nielsen et al., 2010). Despite this variation, the depth of O₂ penetration is generally shallow and
307 was never more than 25% of the thickness of the crust (Nielsen et al., 2010). The restriction of significant O₂
308 penetration is likely due to surface crusts being a floating organic structure on top of liquid slurry, where the
309 bottom of the crust is always saturated, while aerobic processes actively consume O₂ in upper layers. Nielsen
310 et al. (2010) proposed that trapping of gases formed in the slurry could elevate the crust above the liquid
311 slurry phase and, as a result, improve O₂ penetration. Measurements of O₂ at a fixed depth over 48 h did
312 indicate gas pockets that lifted the crust, but they were intermittently deflated (Supplemental Fig. S3).
313 Structural voids in the crusts, such as pores and crevices, could also provide access for O₂ to deeper parts of
314 the crusts, and weather conditions such as precipitation and drought will influence O₂ permeability by
315 altering the wetness of the crust (Hansen et al., 2009).

316 The present study observed N₂O levels as high as 100 µmol L⁻¹, which was far above the N₂O concentrations
317 commonly found in other environments. For example, Baral et al. (2014) and Zhou et al. (2016) reported N₂O
318 concentrations of < 5 µmol L⁻¹ near the soil surface. Careful examination of potential interferences to the
319 microsensor is therefore warranted. Surface crusts may contain up to 300 µmol L⁻¹ of H₂S (Nielsen et al.,
320 2010), and H₂S is known to affect the signal of N₂O microsensors. However, the microsensor used here is
321 equipped with an alkaline oxygen guard which converts incoming H₂S to ionic forms, and this offers some
322 protection to the cathode from H₂S (Andersen et al., 2001). With the same type of microsensor used in this
323 study, Andersen et al. (2001) found a reduction in the sensitivity towards N₂O with increasing concentrations
324 of H₂S up to 350 µmol L⁻¹, indicating that any H₂S interference would result in lower, not higher, N₂O
325 readings. Therefore, the high N₂O concentrations observed were not likely to be a result of H₂S interference.
326 In the above mentioned studies where soil N₂O concentrations were <5 µmol L⁻¹, the corresponding N₂O
327 emissions were 3.6 and 25 µmol m⁻² h⁻¹ (Baral et al., 2014; Zhou et al., 2016). For livestock slurry with
328 surface crusts, N₂O emissions as high as 393–1,429 µmol m⁻² h⁻¹ have been reported (Hansen et al., 2009;

329 Sommer et al., 2000). Considering the relationship between N₂O concentration and emission, N₂O
330 concentrations up to 100 μmol L⁻¹ in surface crusts seem plausible.

331 Maximum N₂O accumulation occurred near the oxic-anoxic interface in most cases, and therefore both
332 nitrification and denitrification were potential sources of N₂O (Braker and Conrad, 2011). Law et al. (2012)
333 found a correlation between N₂O production and ammonia oxidation rate, possibly as a result of nitrifier
334 denitrification to conserve O₂ or prevent NO₂⁻ toxicity (Lawton et al., 2013), and similar mechanisms could
335 lead to N₂O accumulation via ammonia oxidation in surface crusts. At or below oxic-anoxic interfaces,
336 incomplete heterotrophic denitrification was likely the main source of N₂O due to intolerance of N₂O
337 reductase towards trace O₂ (Thomson et al., 2012). Nitrous oxide could also be released as a terminal
338 product from methane oxidation coupled with partial denitrification by *M. denitrificans* (Kits et al., 2015b)
339 and *M. album* (Kits et al., 2015a; Nyerges et al., 2010), which can be enabled by hypoxia developed at the
340 oxic-anoxic interfaces. Denitrification could also act as a sink for NO₃⁻ or, particularly, NO₂⁻ which is a strong
341 inhibitor of aerobic methane oxidation. The ubiquitous presence of N₂O in the surface crusts indicated active
342 transformations of N species which could interfere with methane oxidation. Yet, as seen from the incubation
343 experiments, the effect of N species on MOB may be either inhibitory or stimulatory depending on other
344 factors. Moreover, Type I MOB utilizing NO₂⁻ and/or NO₃⁻ for methane oxidation are presumably more
345 resilient to N inhibition than others (Zhu et al., 2016). In support of this, *M. denitrificans* and *M. album* have
346 been shown to tolerate and grow under 10 mM NO₃⁻ (Kits et al., 2015b) and 2.5 mM NO₂⁻ (Nyerges et al.,
347 2010), respectively. Also, Hu and Lu (2015) found that, while NH₄⁺ and NO₃⁻ both stimulated Type I MOB as
348 determined from *pmoA* gene copy numbers, Type II MOB were inhibited by NH₄⁺ as concentrations
349 increased.

350 Clearly, various physio-chemical and biological properties are involved in regulating methane oxidation in
351 surface crust. Firstly, the heterogeneous structure of the crust adds complexity to the distribution of gases.
352 Secondly, there are complex interactions between CH₄ and O₂ levels, and inorganic N species and
353 concentration, with respect to methanotrophic activity. In parts of the crust where CH₄ availability is

354 relatively low, moderate O₂ limitation probably has little impact on aerobic methane oxidation activity,
355 whereas N inhibition could be significant depending on N species and concentration. In contrast, in parts of
356 the crust with high CH₄ availability, O₂ limitation is likely the main control of aerobic methane oxidation, and
357 inhibition due to inorganic N is only important where O₂ is not limiting.

358 ***Conclusions***

359 Microsensor measurements of *in situ* O₂ and N₂O profiles revealed shallow penetration of O₂ into slurry
360 surface crusts and active N transformations around oxic-anoxic interfaces. Laboratory incubations suggested
361 that O₂ availability was more important to high-concentration than low-concentration methane oxidation,
362 and there were complex interactions between inorganic N and O₂ limitation. The incorporation of ¹³C from
363 CH₄ into membrane PLFAs indicated that both Type I and Type II MOB were actively involved in methane
364 oxidation, but with Type I MOB dominating the activity at high CH₄ concentrations. These observations
365 together imply that manipulation of storage conditions to increase headspace CH₄ concentration, as
366 proposed by Petersen and Miller (2006), could stimulate methane oxidation by Type I MOB in the upper
367 parts of the crust where O₂ is non-limiting and mineral N availability low.

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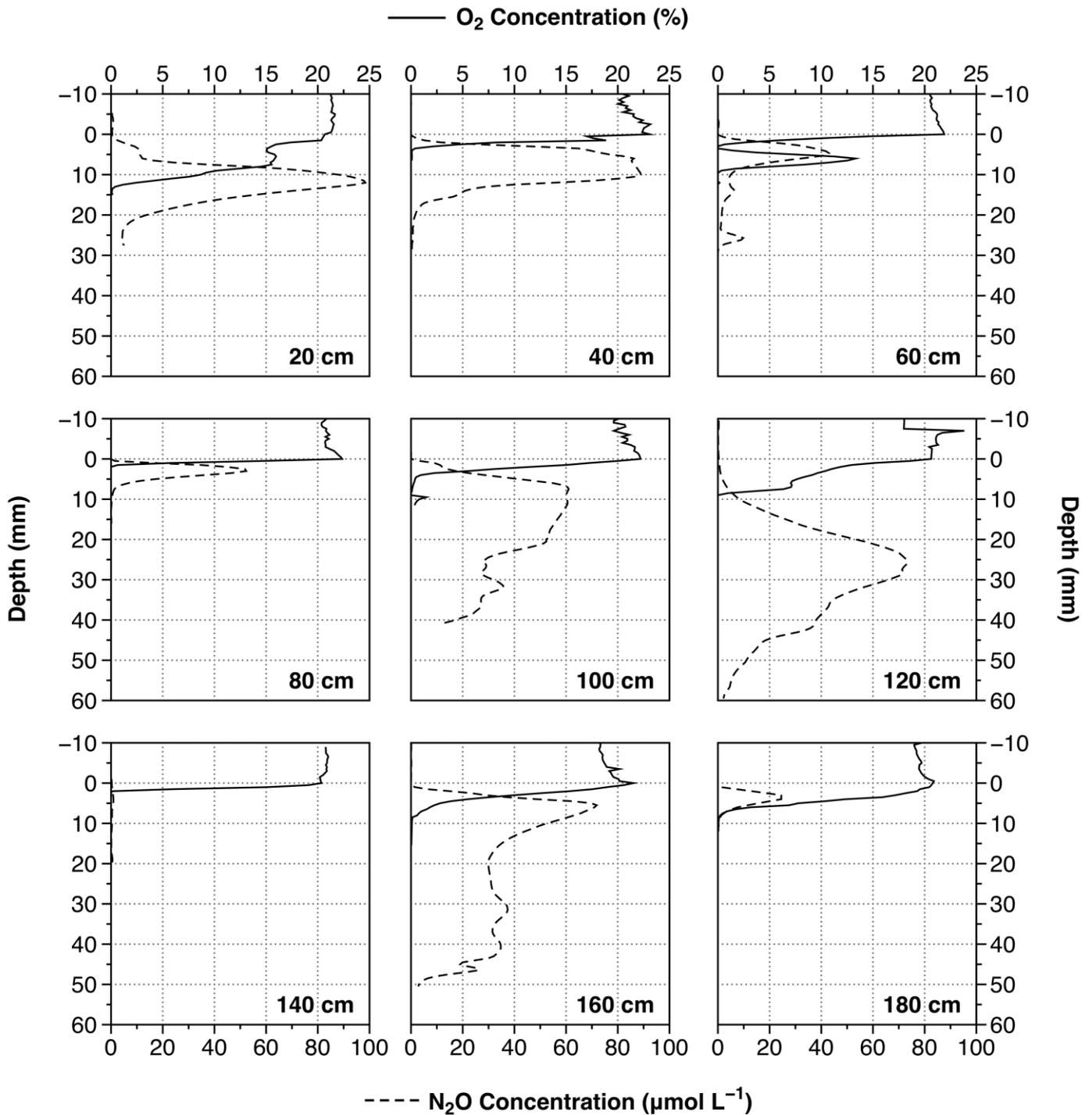


Figure 1: Profiles of oxygen (O₂, solid line, top X-axis) and nitrous oxide (N₂O, dashed line, bottom X-axis) profiles in a surface crust measured using microsensors at 20 cm intervals along the 200-cm diameter of a slurry storage tank. Distances of the sampling points from the edge of the tank are indicated on the bottom-right corner of each panel. Oxygen was traced to a depth of 30 mm in all cases; N₂O was traced to a depth of 30 mm at 20–80 cm and to a depth of 60 mm at 100–180 cm.

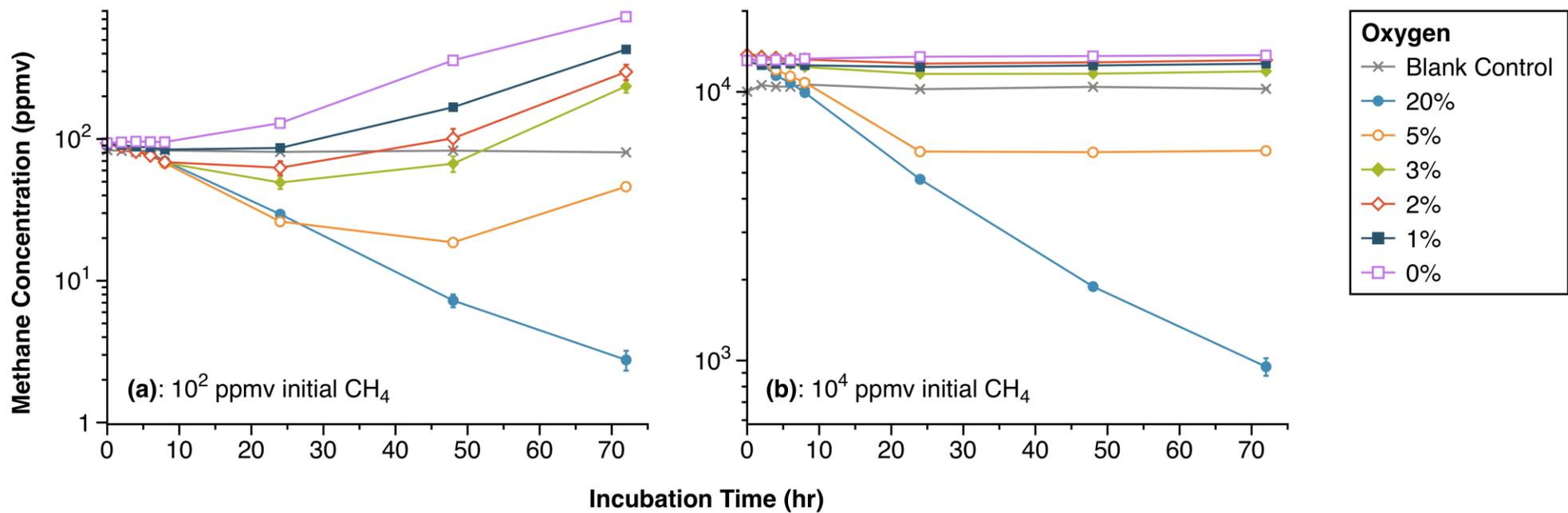


Figure 2: Dynamics of CH₄ concentrations during a 72-h incubation of slurry surface crusts with initial O₂ concentrations of 20, 5, 3, 2, 1, and 0%, and with initial CH₄ concentrations of 10² ppmv (a) and 10⁴ ppmv (b). Blank control contained 20% initial headspace O₂ and no crust material. Each point represents the mean of triplicate assays, and error bars show standard error.

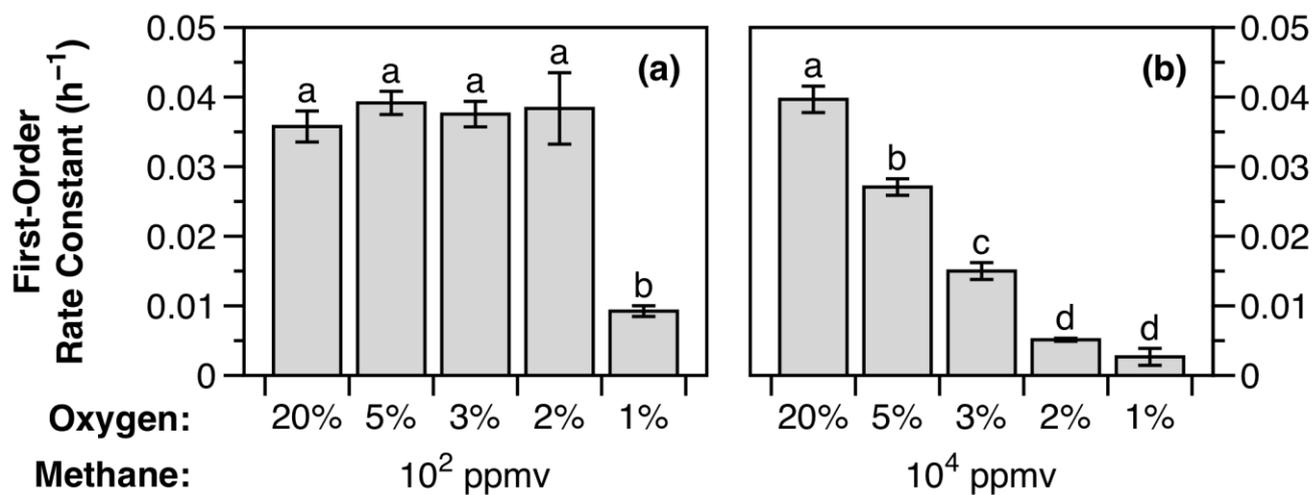


Figure 3: First-order rate constants of potential CH₄ oxidation under different O₂ and CH₄ concentrations during the first 8 h of incubation. The bars show the mean of triplicate assays, and the error bars show standard error. For each CH₄ concentration, bars denoted with the same letter on top are not significantly different at $\alpha = 0.05$.

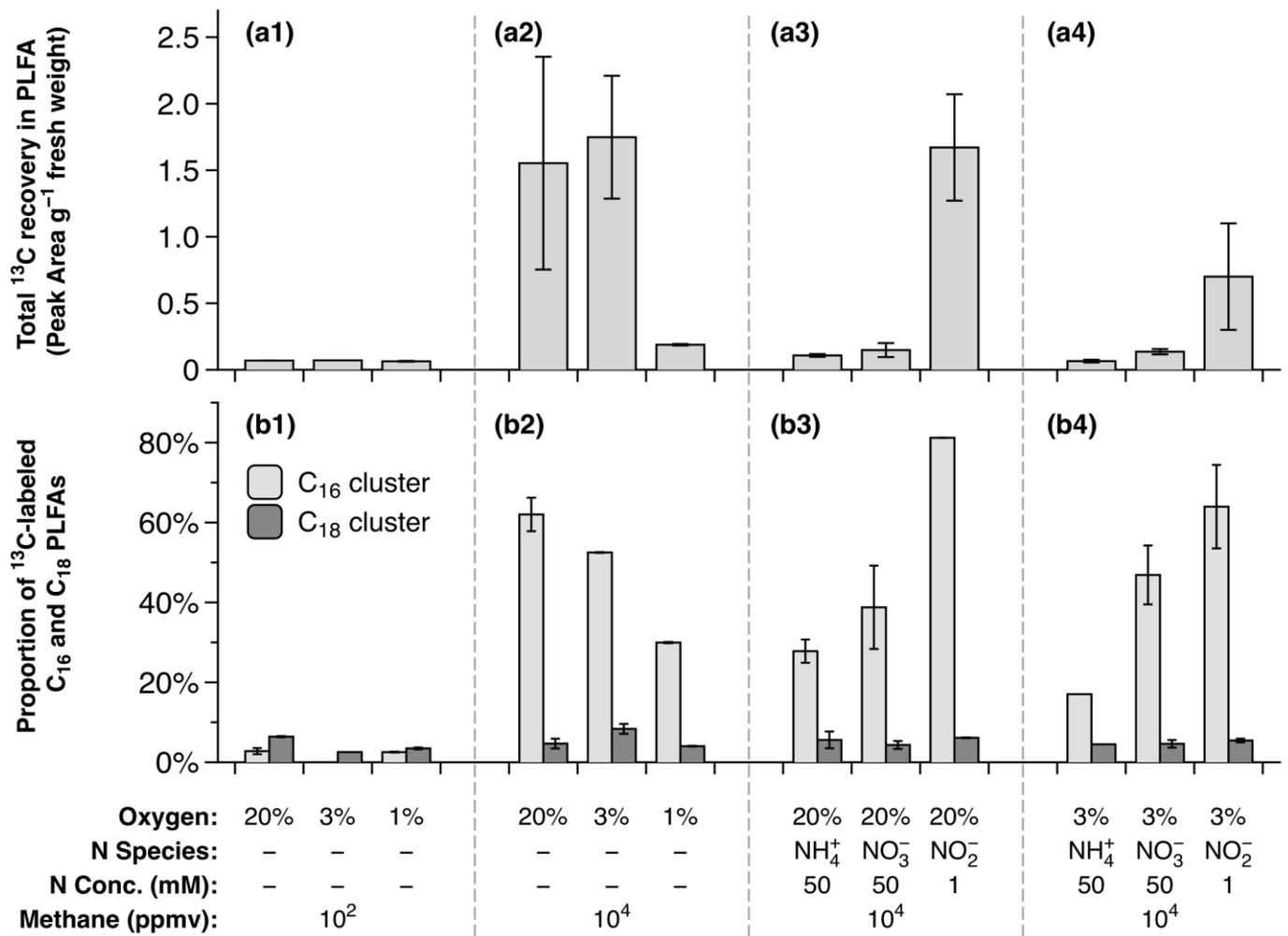


Figure 4: Total recovery of ^{13}C in PLFA (expressed as peak area per gram fresh crust) derived from incubation of surface crusts with $^{13}\text{CH}_4$ (a1–a4), and proportions of ^{13}C incorporation into PLFA belonging to C_{16} or C_{18} clusters (b1–b4) at different combinations of O_2 , CH_4 , and inorganic N availability. Data shown are means and standard errors of two replicates.

Table 1: Selected chemical properties of various livestock slurries and surface crusts from previous studies.

Sample	Surface Crust			Slurry	
	NH ₄ ⁺ μmol kg ⁻¹ WW*	NO ₃ ⁻ μmol kg ⁻¹ WW	NO ₂ ⁻ μmol kg ⁻¹ WW	pH	NH ₄ ⁺ mM
Cattle slurry surface crusts developed for 3 years (Duan et al., 2013)	116.3	370.9	n.a. [#]	n.a.	n.a.
Various cattle and swine slurries and surface crusts (Nielsen et al., 2010)	n.a.	7– 3,602	2–98,000	6.96–7.7	84–205
Surface crusts of various dryness (Hansen et al., 2009)	n.a.	290–35,000	80–1,990	7.7	175
Various cattle and swine slurries (Sommer et al., 2007)	n.a.	n.a.	n.a.	n.a.	79–257
Surface crusts (Petersen et al., 2006)	1,432–54,923	91–16,378	3–50	7.13–8.89	1.7–69
Digested cattle slurry (Clemens et al., 2006)	n.a.	n.a.	n.a.	7.4–7.8	85–127

* WW, wet weight.

[#] n.a., value not reported.

Table 2: Combinations of CH₄, O₂, and inorganic N amendments used in this study. The values indicate the number of replicates prepared. Treatments marked with asterisks (*) were used for analysis of ¹³C-labelled phosphate lipid fatty acids (PLFAs).

N Species and Concentrations	CH ₄ : 10 ² ppmv							10 ⁴ ppmv					
	O ₂ :	0%	1%	2%	3%	5%	20%	0%	1%	2%	3%	5%	20%
None		3	3*	3	3*	3	3*	3	3*	3	3*	3	3*
NH ₄ ⁺	10 mM				2		2				2		2
	50 mM				2		2				2*		2*
NO ₃ ⁻	10 mM				2		2				2		2
	50 mM				2		2				2*		2*
NO ₂ ⁻	1 mM				2		2				2*		2*

Table 3: First-order rate constants (h^{-1}) of CH_4 oxidation in slurry surface crust samples under different O_2 concentrations and N amendments. Numbers in parentheses indicate relative activity (the ratio of the treatment activity as compared to the activity of the N-free control). Under each CH_4 concentration, values followed by the same letter are not significantly different at $\alpha = 0.05$.

N Species and Concentrations		First-Order Rate Constants (h^{-1})			
		10^2 ppmv CH_4		10^4 ppmv CH_4	
		20% O_2	3% O_2	20% O_2	3% O_2
None		0.036 (1.00) a	0.037 (1.00) a	0.040 (1.00) b	0.016 (1.00) e
NH_4^+	10 mM	0.015 (0.42) d	0.010 (0.27) ef	0.031 (0.78) c	0.017 (1.06) e
	50 mM	0.007 (0.19) fg	0.005 (0.14) g	0.013 (0.33) e	0.005 (0.31) f
NO_3^-	10 mM	0.029 (0.81) b	0.022 (0.59) c	0.054 (1.35) a	0.022 (1.38) d
	50 mM	0.017 (0.47) d	0.013 (0.35) de	0.028 (0.70) c	0.021 (1.31) d
NO_2^-	1 mM	0.008 (0.22) fg	0.008 (0.22) fg	0.016 (0.40) e	0.017 (1.06) e

Methanotrophic activity in slurry surface crusts as influenced by CH₄, O₂, and inorganic N

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(a). The 200-cm long aluminum bar with distance marks.



(b). The aluminum bar installed on the inner rim of the slurry tank.



(c). The motorized micromanipulator mounted on the aluminum bar.



(d). The microsensor fixed to the micromanipulator.

Figure S1: Custom-made mounting system for *in situ* measurement of O₂ and N₂O profiles using microsensors.

A 200-cm long aluminum bar with mm-scale distance marks (a) was installed on the inner rim of the storage tank, approximately 40 cm above the surface crust (b). A computer-controlled motorized micromanipulator (Unisense, Aarhus, Denmark) capable of vertical movement was mounted on a custom-made rack, which could be moved manually along the length of the aluminum bar (c). The microsensor was fixed to the micromanipulator using a rubber-lined clamp (d). When installed at the initial position for measurement, the tip of the microsensor was approximately 1 cm above the surface of the crust. During measurements, a cover was placed loosely over the storage tank to avoid heating of the surface crust by direct insolation.

When measurement at one sampling point was completed, the microsensor was retreated to initial position and temporarily detached from the micromanipulator to protect the tip from breaking during movement, and reinstalled after the micromanipulator had been moved to the next sampling point.

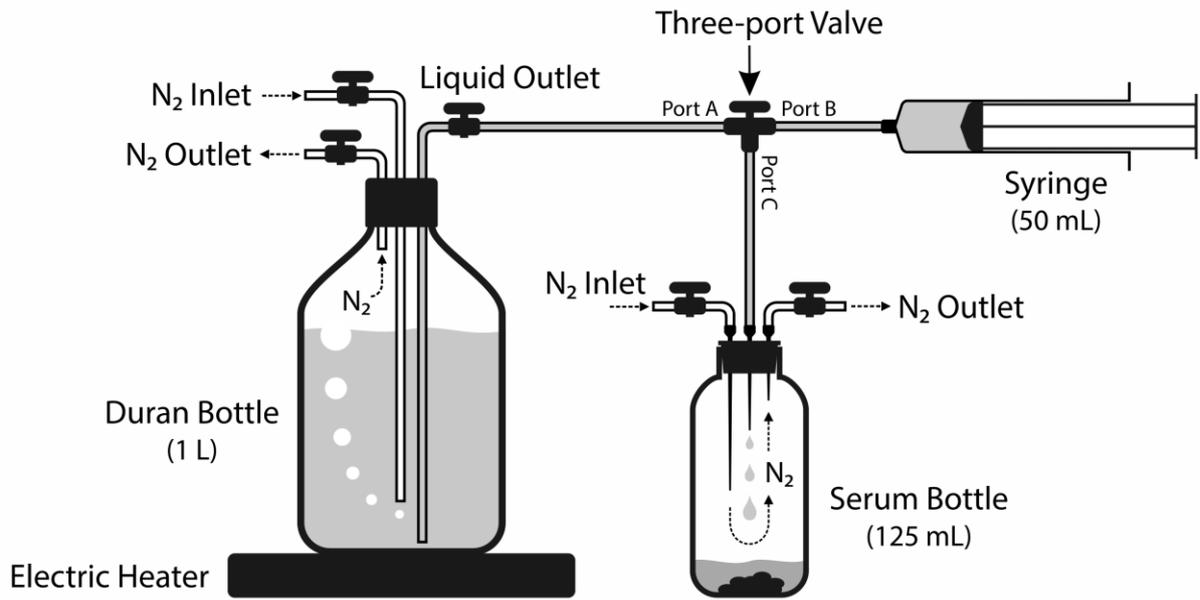


Figure S2: Diagram of the setup to prepare and distribute anoxic incubation medium.

A Duran bottle was modified to have three plastic tubes inserted through the cap: one as N₂ inlet, one as N₂ outlet, and the third as liquid outlet. The Duran bottle was filled with BS medium to ca. 70% of its volume and placed on an electric heater with the cap tightly secured. The BS medium was heated to the boiling point while bubbled with pure N₂ for at least 30 min, and then cooled to room temperature still under N₂ bubbling. Then, the N₂ outlet was closed and the liquid outlet valve was opened, and the entire tubing was flushed and filled with anoxic BS medium by the build-up of gas pressure inside the Duran bottle. When distributing BS medium to the serum bottle, a needle connected to pure N₂ flow was first inserted into the serum bottle through the rubber stopper and then, a second needle was inserted as N₂ outlet. The serum bottle was flushed with N₂ for 10 sec, and then a third needle connected to the three-port valve was inserted. Ports A and B of the three-port valve were opened while C was closed, and the syringe was filled with 20 mL incubation medium. Then, ports B and C were opened and A was closed and the incubation medium was injected from the syringe into the serum bottle while the bottle was being flushed by pure N₂.

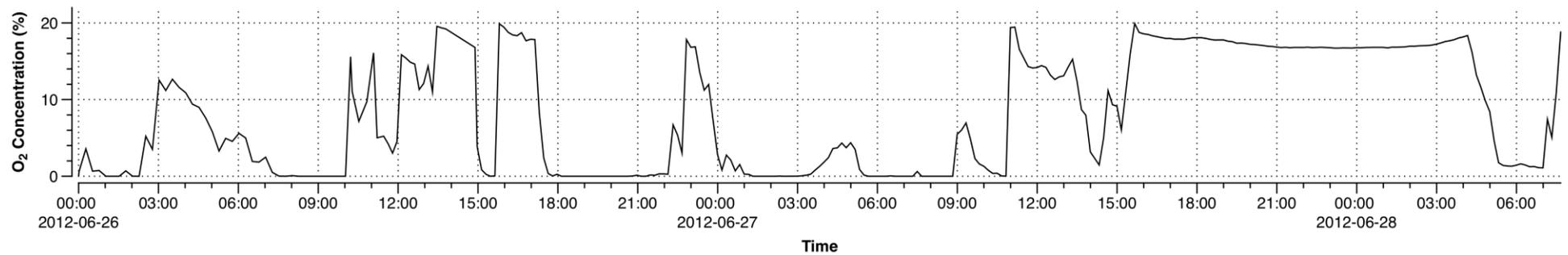


Figure S3: Dynamics of O₂ concentration at a fixed depth in a surface crust over 48 hours. The storage tank was covered to eliminate wind effect but was passively ventilated. The microsensor was initially fixed in a position where the tip just touched the surface of the crust. Oxygen concentrations were measured every 10–15 min as described in the manuscript. The peaks in O₂ profile showed that the microsensor was alternately exposed to atmospheric O₂ and more anaerobic conditions. This indicated that the surface of the crust periodically rose up and moved down, likely due to inflation and deflation of gas pockets underneath the crust.

Table S1: Oxygen Consumption during CH₄ oxidation at 10,000 ppmv initial CH₄.

Initial State (time 0)		After 8 h		
Headspace O ₂ (%)	Dissolved O ₂ (μM)	CH ₄ Consumed (μM)	O ₂ Consumed (μM)	O ₂ Remaining (μM)
20	256.0	4.92	9.84	246.2
3	38.4	1.95	3.90	34.5
1	12.8	0.57	1.14	11.7

- Stoichiometry of CH₄ oxidation is:

$$\text{CH}_4 + (2 - x)\text{O}_2 \longrightarrow (1 - x)\text{CO}_2 + x\text{CH}_2\text{O} + (2 - x)\text{H}_2\text{O}$$
 where x is the fraction of carbon that is assimilated into biomass (CH₂O) (Urmann et al., 2007). Therefore, CH₄ to O₂ ratio in CH₄ oxidation theoretically ranges between 1:1 (100% C assimilation) and 1:2 (no C assimilation). In this calculation, we assumed maximum O₂ consumption, i.e. a CH₄ to O₂ ratio of 1:2.
- Henry's Law constant (K) for O₂ and CH₄ are 1.28 and 1.34 mmol L⁻¹ atm⁻¹, respectively.
- Dissolved O₂ was calculated as: $K \times [\text{Headspace O}_2]$.
- Diffusivities of dissolved O₂ and CH₄ in water at 20°C are 2.06 and 1.75×10^{-5} cm² sec⁻¹, respectively (Broecker and Peng, 1974). Therefore, there's no diffusion limit of O₂ for CH₄ oxidation.