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Methanotrophic activity in slurry surface crusts as influenced by CH\(_4\), O\(_2\), 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.
Abstract

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

Introduction

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

Methane oxidation potential depends on CH₄ and O₂ availability, which are highly variable due to the heterogeneous structure of the crust. Since surface crusts overlie liquid manure with a high methanogenic potential, they are typically high-CH₄ environments with concentrations far above the atmospheric level. We have observed up to 200 ppmv CH₄ in the stagnant atmosphere immediately above the surface crust (Y.F. Duan, unpublished data), and headspace CH₄ concentrations of 10² and 10⁴ ppmv were used previously to simulate this range of CH₄ availability for MOB in laboratory incubations (Duan et al., 2013). Due to the often loose structure of the crust, sectioning for extraction and determination of in situ CH₄ availability by sampling are impractical. While a CH₄ biosensor has been described (Damgaard and Revsbech, 1997), it is not commercially available and has not been tested in heterogeneous environments such as surface crusts.

In contrast to CH₄, the distribution of O₂ in surface crusts can be readily determined using a microsensor (Revsbech, 2005), as demonstrated by Hansen et al. (2009) and Nielsen et al. (2010) who investigated O₂ penetration into various slurry crusts.

Inhibition of methane oxidation by inorganic N is known from many environments (Bosse et al., 1993; Dunfield and Knowles, 1995; Wang and Ineson, 2003), as well as in surface crusts (Duan et al., 2013), but stimulation or no effect can also occur (Liu and Greaver, 2009). Livestock slurry and surface crusts are highly enriched in inorganic N (Table 1): the slurry phase may contain up to 200 mM ammoniacal N (NH₃/NH₄⁺) (Nielsen et al., 2010; Sommer et al., 2007), while up to 35 mM NO₃⁻ (Hansen et al., 2009) and 98 mM NO₂⁻ (Nielsen et al., 2010) have been found at the oxic-anoxic interface in surface crusts. These concentration ranges (Table 1) represent a high variability of inorganic N at microsites within surface crusts due to fluctuations in water content as a result of precipitation and insolation (Nielsen et al., 2010). Due to this heterogeneity, bulk N concentrations are insufficient to characterize N distribution and thus potential interference with methane oxidation in microsites. While specialized sensors are available to determine micro-scale concentrations of NH₄⁺, NO₂⁻, and NO₃⁻ in aqueous environments (De Beer et al., 1997; De Beer
et al., 1991; Larsen et al., 1996), they are difficult to apply in unsaturated matrices such as surface crusts, where contact to liquid phase may be intermittent. Instead, the accumulation of N$_2$O in the crust was used in this study as a proxy for the distribution of N transformations and thus presence of inorganic N (Larsen et al., 1996), as both nitrification and denitrification can produce N$_2$O under sub-oxic conditions (Bollmann and Conrad, 1998).

The effects of environmental factors on methane oxidation in surface crusts will ultimately depend on the MOB present (Bodelier, 2011; Hu and Lu, 2015). Aerobic MOB have been conventionally categorized into Type I and Type II based on phylogenetic and functional traits, including the presence of signature 16-carbon (C$_{16}$) or 18-carbon (C$_{18}$) phospholipid fatty acids (PLFAs) (Hanson and Hanson, 1996). Type I MOB have been reported to thrive in N-sufficient, high-O$_2$ and low-CH$_4$ environments, whereas Type II MOB seem to favor the opposite (Amaral et al., 1995; Amaral and Knowles, 1995; Graham et al., 1993). It is still unclear to what extent CH$_4$, O$_2$, and inorganic N conditions will select one type of MOB over another in surface crusts, but previous studies found that Type I MOB are more abundant and diverse than Type II MOB in this environment (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2013). In recent years, novel methane oxidation pathways, such as NO$_2^-$ dependent anaerobic methane oxidation (Ettwig et al., 2010; Welte et al., 2016), as well as aerobic methane oxidation coupled with partial denitrification (Kits et al., 2015a; Kits et al., 2015b), have also been described, but the importance of these processes and the presence of relevant microorganisms in surface crusts remain unclear.

A main objective of this study was to examine the potential for microbial methane oxidation under realistic storage conditions by characterizing in situ distributions of O$_2$ and inorganic N transformations in a cattle slurry surface crust using microsensors. Effects and interactions of O$_2$ and inorganic N species with respect to methane oxidation could not be quantified in situ where microbial activities occur in micro-sites, and instead this was investigated under controlled laboratory conditions. Here, $^{13}$CH$_4$ was used as substrate, allowing $^{13}$C stable isotope probing of PLFAs to study the involvement of Type I and Type II MOB in methane oxidation in surface crusts. Based on previous results (Duan et al., 2014; Duan et al., 2013) we hypothesized that
methane oxidation activity in surface crusts would be determined by both \( \text{CH}_4 \) and \( \text{O}_2 \) availability, and inhibited by inorganic \( \text{N} \), and that Type I MOB would be primarily responsible for methane oxidation.

**Materials and Methods**

**Microsensor Measurement of \( \text{O}_2 \) and \( \text{N}_2\text{O} \) Distribution**

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

Oxygen and \( \text{N}_2\text{O} \) concentration profiles in the surface crust were determined using, respectively, an \( \text{O}_2 \) and a \( \text{N}_2\text{O} \) microsensor with a tip diameter of 0.5 mm (both produced by Unisense, Aarhus, Denmark). Both microsensors were calibrated according to manufacturer’s instructions. Detection limits for \( \text{O}_2 \) and \( \text{N}_2\text{O} \) were 0.3 \( \mu \text{mol L}^{-1} \) and 0.1 \( \mu \text{mol L}^{-1} \), respectively.

A custom-made mounting system was used to place the microsensors over the surface crust (Supplemental Fig. S1). Oxygen profiles were recorded at 20-cm intervals from 20 to 180 cm along the 200-cm diameter of the storage tank. During measurement, the microsensor was introduced stepwise into the crust at 0.5 mm increments to a maximum depth of 30 mm using a motorized micromanipulator (Unisense). At each depth, the microsensor was stationary for 3 s to allow gas equilibration, and then the \( \text{O}_2 \) concentration was determined as the average of a 3 s reading. The signal was amplified by a multimeter (Unisense), and registered by the SensorTrace PRO v3.0 software (Unisense). Nitrous oxide profiles were determined at the same locations as \( \text{O}_2 \) profiles, but with a +2 mm offset to avoid any disturbance to the crust caused by the \( \text{O}_2 \) microsensor. The \( \text{N}_2\text{O} \) profiles were recorded with the same procedure as \( \text{O}_2 \) profiles, and initially over the
same depth. However, due to incidences of significant N$_2$O accumulation at 30 mm depth, the maximum depth was extended to 60 mm starting from the 5th profile (at 100 cm distance from the edge).

Due to the uneven surface of the crust, a fixed depth cannot accurately define the crust-air interface. For O$_2$, an abrupt decrease from atmospheric concentration defined the crust-air interface. For N$_2$O, which was produced inside the crust and diffused towards the atmosphere, the crust-air interface was defined as the depth where N$_2$O concentration dropped below the detection limit. Using these two criteria, the O$_2$ and N$_2$O concentration profiles were aligned.

**Methane Oxidation in Response to O$_2$ and N Amendments**

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

Methane oxidation rates were determined by incubating 3-g crust samples in liquid media under a controlled atmosphere. To reduce background N, crusts were washed three times by vortexing with 20 mL deionized water followed by centrifugation at 10,000 × g for 10 min. The washing did not remove all NH$_4^+$, but the residual NH$_4^+$ was negligible compared to the received NH$_4^+$ amendment (Duan et al., 2013). Washed crust materials were transferred to 125 mL serum bottles, and resuspended in 20 mL basal salt (BS) medium prepared according to Whittenbury et al. (1970), but excluding N salts. The BS medium was dispensed using a customized system designed to remove dissolved O$_2$ and maintain anoxia during sample preparation (Supplemental Fig. S2).

Various treatments with combinations of different CH$_4$, O$_2$, and inorganic N were prepared (Table 2). For N amendments, pre-made solutions of (NH$_4$)$_2$SO$_4$, KNO$_3$, or KNO$_2$ were injected into the serum bottles to
achieve the desired N concentrations. The bottles were purged using a vacuum pump and refilled with helium, and this step was repeated three times to exhaust residual O₂. Then, air and ¹³C-labeled CH₄ (99 atom% ¹³C, ISOTEC, Miamisburg, OH, USA) were injected to achieve the desired headspace gas concentrations. To meet the requirement for CO₂ by some MOB, 4 mL pure CO₂ was also added (Acha et al., 2002). After gas injection, the bottles were mounted on a rotary shaker at 150 rpm for 30 min to allow for liquid-gas equilibration. Headspace O₂ concentrations were verified using an Agilent 3000A MicroGC (Hørsholm, Denmark) as described by (Petersen et al., 2009). For treatments with 0% O₂, residual headspace O₂ concentration was undetectable (< 10–20 ppmv). The bottles were then incubated on a rotary shaker at 200 rpm at ca. 21°C; Duan et al. (2013) had shown that there is no gas diffusion limitation under these incubation conditions. Headspace CH₄ concentrations were measured after 0, 2, 4, 6, 8, 24, 48, and 72 h using a Shimadzu 14B GC as described by Duan et al. (2013).

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

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

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

After incubation with ¹³C-labeled CH₄, selected crust samples were processed for PLFA analysis (Table 2). Prior to lipid extraction it was necessary to reduce the organic load since otherwise the humic material would bind the chloroform phase and prevent isolation of lipid-soluble compounds. Crust samples were vortexed and centrifuged for 10 min at 3000 × g to extract microbial cells. The supernatant was filtered
through 0.2 μm chloroform-soluble polycarbonate filters, and the material retained on the filter was used for lipid extraction. Hence, lipid results refer to the fraction of extractable low particle size material only.

Polar lipid fatty acid methyl esters (FAMEs) from each filter were prepared as previously described by Petersen et al. (2002).

FAMEs were analyzed using a HP6890 GC (Agilent, Santa Clara, CA, USA) coupled via a GC combustion interface (Thermo Scientific, Bremen, Germany) in continuous flow mode to a Finnigan DeltaPLUS isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The oxidation reactor on the interface was maintained at 940 °C, the reduction reactor at 650 °C. Samples (1 µL) were injected at 240 °C in splitless mode. The column temperature was held at 50 °C for 2 min, then increased at 15 °C min⁻¹ to 100 °C, subsequently at 2 °C min⁻¹ to 220 °C, and finally at 15 °C min⁻¹ to 240 °C, where the final temperature was held for 5 min. Separated compounds were measured against a CO₂ reference gas calibrated with reference to Vienna PeeDee belemnite. PLFAs were identified by relative retention time comparing samples against a FAME standard mix (Supelco 37 component FAME mix, 47885-U, Sigma Aldrich). All δ¹³C values were corrected for the methanol C added during methanolysis:

\[ \delta^{13}C_{\text{FAME}} = \frac{(N_{FA} + 1)\delta^{13}C_{\text{FAME}} - \delta^{13}C_{\text{MeOH}}}{N_{FA}} \]

where \( N_{FA} \) refers to the number of carbon atoms of the fatty acid component, \( \delta^{13}C_{\text{FAME}} \) is the observed δ¹³C value of the FAME, and \( \delta^{13}C_{\text{MeOH}} \) is the δ¹³C value of the methanol used for methanolysis (−37.7‰ ± 3.2‰). The δ¹³C isotope ratios were converted to atom%, and atom% excess was then calculated by subtraction of an unlabeled control. The incorporation of \(^{13}\text{CH}_4 \) into membrane PLFAs (\( n_{13C}, \text{nmol} \)) was calculated as \( n_{13C} = (P_{\text{FAME}} / P_{\text{ISME}}) \times n_{\text{ISME}} \times (\text{atom% } ^{13}\text{C excess}) \), where \( P_{\text{FAME}} \) and \( P_{\text{ISME}} \) are peak areas of the FAME and internal standard \( \text{Me19:0} \), respectively, and \( n_{\text{ISME}} \) (nmol C) is the concentration of the internal standard fatty acid. The lower limit of identified peaks corresponded to 0.1 ng \(^{13}\text{C} \) g⁻¹ crust material.
In view of the uncertain recovery of microbial cells following centrifugation and filtration, statistical testing of treatment effects was not performed, and results will only be presented as means ± standard errors.

Results

Oxygen and N₂O Distribution in Natural Surface Crusts

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

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

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

Response of CH₄ Oxidation to O₂ Concentrations

Figure 2 shows the changes in headspace CH₄ concentrations during the 72-h incubation study with different initial O₂ levels and two initial CH₄ levels. For both 10² and 10⁴ ppmv CH₄, consistent methane consumption throughout the incubation was observed only at 20% initial O₂, with a > 90% decline in headspace CH₄ concentrations after 72 h. In treatments with ≤ 5% initial O₂, CH₄ consumption was generally observed within the first 24 h, followed by net CH₄ accumulation. At 0% initial O₂, net CH₄ production was observed throughout the incubation.
The strength of methane oxidation activity was expressed as first-order rate constants during the first 8 h of incubation (Fig. 3). At $10^2$ ppmv initial CH$_4$ there were no significant differences in first-order rate constants 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$_4$, methane oxidation rates were significantly reduced at lower O$_2$ levels, though not significantly different between 2% and 1% O$_2$.

### Response of CH$_4$ Oxidation to Interactions between O$_2$ and Inorganic N

When samples with manipulated O$_2$ and CH$_4$ concentrations were amended with inorganic N, complex patterns in microbial methane oxidation were observed. For each of the two initial CH$_4$ concentrations, two-way ANOVA showed significant effects of O$_2$ concentrations and N amendments, as well as their interaction, on methane oxidation rates.

At $10^2$ ppmv CH$_4$, first-order rate constants in all N-amended samples were lower than in the N-free control (Table 3). The inhibition was strengthened at increasing concentrations of both NH$_4^+$ and NO$_3^-$. Samples treated with NO$_3^-$ consistently showed less inhibition than treatments receiving other N salts, whereas NO$_2^-$ was a potent inhibitor as indicated by similar inhibitions with 1 mM NO$_2^-$ and 50 mM NH$_4^+$. At 3% O$_2$, inhibition by individual N species and concentrations was slightly stronger than at 20% O$_2$, but the difference was not always statistically significant.

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$_3^-$ as the strongest inhibitor (Table 3). Yet, several NO$_3^-$ treatments caused a weak stimulation rather than inhibition as compared to the N-free control. Generally, the N amendments inhibited methane oxidation at 20% O$_2$, but not at 3% O$_2$, where low rates were already observed in the N-free control. One exception, though, was the inhibition caused by the treatment with 50 mM NH$_4^+$. 
13C Incorporation into C16 and C18 PLFAs

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

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

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

Discussion

Due to the heterogenous nature of surface crusts, microbial activities within this environment are controlled by physical and chemical properties of individual microsites rather than overall bulk properties. However, detailed analysis of surface crusts is challenged by the often loose and fibrous structure of the material, and this was also the case with the straw-containing cattle slurry crust used in this study. Therefore, we chose to
characterize O$_2$ and inorganic N distributions \textit{in situ} by microsensors, while regulation of microbial activities were investigated by controlled laboratory incubations. For logistic reasons, the \textit{in situ} gas measurements and laboratory incubations were performed using different surface crusts. However, previous studies have shown that crusts of different origin are qualitatively similar with respect to, e.g., depth of O$_2$ penetration and the presence of MOB (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2010; Nielsen et al., 2013), and results from the two parts are therefore analyzed and discussed together.

**Effects of O$_2$ Limitation and N Amendments**

Methane oxidation kinetics are complex as the reaction involves two substrates, CH$_4$ and O$_2$ (Cai and Yan, 1999). We were not able to monitor O$_2$ concentrations during incubation, but instead calculated O$_2$ consumption by MOB based on the amounts of CH$_4$ consumed, the stoichiometry of methane oxidation (Urmann et al., 2007), and the diffusion coefficients of CH$_4$ and O$_2$ in water (Broecker and Peng, 1974) (Supplemental Table S1). These calculations suggested that there was no diffusional limitation of O$_2$ for methane oxidation, and that the amounts of O$_2$ used for methane oxidation were $< 10\%$ of the available O$_2$ even at 1\% initial O$_2$. Thus, depletion of O$_2$ during incubation must have been mainly due to aerobic processes other than methane oxidation, i.e., any O$_2$ limitation for MOB activity reflected competition for O$_2$ against other aerobes. Headspace CH$_4$ concentrations decreased exponentially during the first 8 h, indicating that the rates of CH$_4$ uptake depended mainly on CH$_4$ availability. Therefore, the reaction was approximated by first-order reaction kinetics, which have also been used previously in studies of microbial methane oxidation (De Visscher et al., 1999; King and Schnell, 1994; King and Schnell, 1998; Petersen and Ambus, 2006). The apparent first-order kinetics suggest that the rate of CH$_4$ uptake, and thus demand for O$_2$, was proportional to the CH$_4$ concentration, which explains our observation that methane oxidation became more affected by O$_2$ limitation at 10$^4$ ppmv than at 10$^2$ ppmv CH$_4$ (Fig. 3).

The neutral to alkaline pH of slurry (Nielsen et al., 2010; Petersen and Ambus, 2006) suggests the presence of free ammonia (NH$_3$), which is a competitive inhibitor for methane oxidation (Carlsen et al., 1991; Gulledge...
and Schimel, 1998). In both the present (Table 3) and a previous study (Duan et al., 2013), inhibition by ammonia was less at $10^4$ compared to $10^2$ ppmv CH$_4$, suggesting that competitive inhibition is important for effects of ammonia. The observations that NO$_3^-$ was a more potent inhibitor of methane oxidation than NO$_2^-$ was also consistent with the report by Duan et al. (2013), where the concentration of NO$_3^-$ resulting in 50% inhibition was 100-fold higher than that of NO$_2^-$. It is likely that these N species both inhibit methane oxidation via nitrite toxicity (Stein and Klotz, 2011).

We further tested interactions of N inhibition with O$_2$ availability by comparing relative activities. If there were an interaction between O$_2$ and inorganic N, a given N amendment would result in different degree of inhibition at 20% and 3% O$_2$ concentrations. At 10$^3$ ppmv initial CH$_4$, 10 mM, but not 50 mM, NH$_4^+$ or NO$_3^-$ amendments caused a stronger inhibition at 3% than at 20% O$_2$ (Table 3), confirming an interaction between N inhibition and O$_2$ limitation at low N concentrations. At 10$^4$ ppmv CH$_4$, the fact that N amendments generally inhibited methane oxidation at 20% O$_2$ but not further at 3% O$_2$ suggested that high-concentration methane oxidation is more sensitive to O$_2$ limitation rather than to N inhibition.

Generally, as an essential substrate for aerobic methane oxidation, O$_2$ has a direct and immediate effect on aerobic methanotrophic activity. On the other hand, the mechanism of N inhibition on methane oxidation is much more complex and may include immediate toxicity to cell growth and enzyme synthesis, as well as delayed influence on microbial community composition (Bodelier and Laanbroek, 2004). The mechanisms behind interactions between controlling factors in surface crusts could not be explained with the data presented here, and more research is needed to further elucidate this matter.

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

Type I and Type II MOB produce unique membrane PLFAs, 16:1$\omega$8 and 18:1$\omega$8, respectively (Bodelier et al., 2009; Hanson and Hanson, 1996). These signature PLFAs are not always present, or present only in low amounts. However, there is also a general predominance of C$_{16}$ and C$_{18}$ PLFAs among Type I and Type II MOB, respectively (Bodelier et al., 2009), and this has been used to evaluate sources of methane oxidation.
by stable isotope probing (Qiu et al., 2008). In the present study, both types of MOB were active, with Type I MOB dominating the methanotrophic activity especially where CH₄ availability was high (Fig. 4, b1 and b2). Molecular analyses of microflora in other crust materials likewise suggested that Type I MOB dominated the methanotrophic community in terms of both diversity and abundance (Duan et al., 2014).

Interestingly, the incorporation of ¹³C into PLFAs was high in surface crusts incubated with NO₂⁻ as compared to those with other N species (Fig. 4, a3 and a4), especially considering that crusts incubated with NO₃⁻ had over two-fold higher ¹³CH₄ uptake than NO₂⁻ treatments (data not shown). Roslev et al. (1997) reported that NH₄⁺ decreased C conversion efficiency and increased respiration of C assimilated by MOB, which is consistent with the low ¹³C recovery from NH₄⁺ treatments in this study. However, the mechanism by which NO₃⁻ and NO₂⁻ could interfere with C assimilation for MOB remains unclear. Alternative pathways for CH₄ uptake may have contributed to the particularly high ¹³C assimilation in NO₂⁻ amended crusts. The microorganism Candidatus Methylostroma oxyfera is able to couple anaerobic methane oxidation with nitrite reduction (Ettwig et al., 2010), and may be widespread in natural environments (Ettwig et al., 2009; Wang et al., 2012; Zhu et al., 2012). We recovered 10MeC₁₆₀ in the present study, which is characteristic of the lipid profile of M. oxyfera (Kool et al., 2012). Still, more concrete evidence, such as the recovery of specific gene markers of M. oxyfera (Luesken et al., 2011), is needed to confirm the presence of M. oxyfera in surface crusts. More recently, gammaproteobacterial (Type I) methanotrophs Methylophilus denitrificans and Methylophilus album have been reported to be able to oxidize methane under hypoxia using oxidized nitrogen as electron acceptor (Kits et al., 2015a; Kits et al., 2015b). The involvement of such a process would be consistent with the high proportion of C₁₆ PLFAs recovered from NO₃⁻ and NO₂⁻ treatments, and the genera Methylophilus and Methylophilus are widespread (Knief, 2015) and were indeed present in surface crusts (Duan et al., 2014). However, there was no direct evidence in the present study to evaluate the presence of these specific strains.
**In-situ O\(_2\) and N\(_2\)O Distribution and Implications for CH\(_4\) Oxidation**

Oxygen distribution in surface crusts vary over short distances, as shown in this and other studies (Hansen et al., 2009; Nielsen et al., 2010). Despite this variation, the depth of O\(_2\) penetration is generally shallow and was never more than 25% of the thickness of the crust (Nielsen et al., 2010). The restriction of significant O\(_2\) penetration is likely due to surface crusts being a floating organic structure on top of liquid slurry, where the bottom of the crust is always saturated, while aerobic processes actively consume O\(_2\) in upper layers. Nielsen et al. (2010) proposed that trapping of gases formed in the slurry could elevate the crust above the liquid slurry phase and, as a result, improve O\(_2\) penetration. Measurements of O\(_2\) at a fixed depth over 48 h did indicate gas pockets that lifted the crust, but they were intermittently deflated (Supplemental Fig. S3).

Structural voids in the crusts, such as pores and crevices, could also provide access for O\(_2\) to deeper parts of the crusts, and weather conditions such as precipitation and drought will influence O\(_2\) permeability by altering the wetness of the crust (Hansen et al., 2009).

The present study observed N\(_2\)O levels as high as 100 \(\mu\)mol L\(^{-1}\), which was far above the N\(_2\)O concentrations commonly found in other environments. For example, Baral et al. (2014) and Zhou et al. (2016) reported N\(_2\)O concentrations of < 5 \(\mu\)mol L\(^{-1}\) near the soil surface. Careful examination of potential interferences to the microsensor is therefore warranted. Surface crusts may contain up to 300 \(\mu\)mol L\(^{-1}\) of H\(_2\)S (Nielsen et al., 2010), and H\(_2\)S is known to affect the signal of N\(_2\)O microsensors. However, the microsensor used here is equipped with an alkaline oxygen guard which converts incoming H\(_2\)S to ionic forms, and this offers some protection to the cathode from H\(_2\)S (Andersen et al., 2001). With the same type of microsensor used in this study, Andersen et al. (2001) found a reduction in the sensitivity towards N\(_2\)O with increasing concentrations of H\(_2\)S up to 350 \(\mu\)mol L\(^{-1}\), indicating that any H\(_2\)S interference would result in lower, not higher, N\(_2\)O readings. Therefore, the high N\(_2\)O concentrations observed were not likely to be a result of H\(_2\)S interference.

In the above mentioned studies where soil N\(_2\)O concentrations were <5 \(\mu\)mol L\(^{-1}\), the corresponding N\(_2\)O emissions were 3.6 and 25 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) (Baral et al., 2014; Zhou et al., 2016). For livestock slurry with surface crusts, N\(_2\)O emissions as high as 393–1,429 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) have been reported (Hansen et al., 2009;...
Considering the relationship between $N_2O$ concentration and emission, $N_2O$ concentrations up to 100 $\mu$mol L$^{-1}$ in surface crusts seem plausible.

Maximum $N_2O$ accumulation occurred near the oxic-anoxic interface in most cases, and therefore both nitrification and denitrification were potential sources of $N_2O$ (Braker and Conrad, 2011). Law et al. (2012) found a correlation between $N_2O$ production and ammonia oxidation rate, possibly as a result of nitrifier denitrification to conserve O$_2$ or prevent NO$_2^-$ toxicity (Lawton et al., 2013), and similar mechanisms could lead to $N_2O$ accumulation via ammonia oxidation in surface crusts. At or below oxic-anoxic interfaces, incomplete heterotrophic denitrification was likely the main source of $N_2O$ due to intolerance of $N_2O$ reductase towards trace O$_2$ (Thomson et al., 2012). Nitrous oxide could also be released as a terminal product from methane oxidation coupled with partial denitrification by *M. denitrificans* (Kits et al., 2015b) and *M. album* (Kits et al., 2015a; Nyerges et al., 2010), which can be enabled by hypoxia developed at the oxic-anoxic interfaces. Denitrification could also act as a sink for NO$_3^-$ or, particularly, NO$_2^-$ which is a strong inhibitor of aerobic methane oxidation. The ubiquitous presence of $N_2O$ in the surface crusts indicated active transformations of N species which could interfere with methane oxidation. Yet, as seen from the incubation experiments, the effect of N species on MOB may be either inhibitory or stimulatory depending on other factors. Moreover, Type I MOB utilizing NO$_2^-$ and/or NO$_3^-$ for methane oxidation are presumably more resilient to N inhibition than others (Zhu et al., 2016). In support of this, *M. denitrificans* and *M. album* have been shown to tolerate and grow under 10 mM NO$_3^-$ (Kits et al., 2015b) and 2.5 mM NO$_2^-$ (Nyerges et al., 2010), respectively. Also, Hu and Lu (2015) found that, while NH$_4^+$ and NO$_3^-$ both stimulated Type I MOB as determined from *pmoA* gene copy numbers, Type II MOB were inhibited by NH$_4^+$ as concentrations increased.

Clearly, various physio-chemical and biological properties are involved in regulating methane oxidation in surface crust. Firstly, the heterogeneous structure of the crust adds complexity to the distribution of gases. Secondly, there are complex interactions between CH$_4$ and O$_2$ levels, and inorganic N species and concentration, with respect to methanotrophic activity. In parts of the crust where CH$_4$ availability is
relatively low, moderate O$_2$ limitation probably has little impact on aerobic methane oxidation activity,
whereas N inhibition could be significant depending on N species and concentration. In contrast, in parts of the crust with high CH$_4$ availability, O$_2$ limitation is likely the main control of aerobic methane oxidation, and inhibition due to inorganic N is only important where O$_2$ is not limiting.

**Conclusions**

Microsensor measurements of *in situ* O$_2$ and N$_2$O profiles revealed shallow penetration of O$_2$ into slurry surface crusts and active N transformations around oxic-anoxic interfaces. Laboratory incubations suggested that O$_2$ availability was more important to high-concentration than low-concentration methane oxidation, and there were complex interactions between inorganic N and O$_2$ limitation. The incorporation of $^{13}$C from CH$_4$ into membrane PLFAs indicated that both Type I and Type II MOB were actively involved in methane oxidation, but with Type I MOB dominating the activity at high CH$_4$ concentrations. These observations together imply that manipulation of storage conditions to increase headspace CH$_4$ concentration, as proposed by Petersen and Miller (2006), could stimulate methane oxidation by Type I MOB in the upper parts of the crust where O$_2$ is non-limiting and mineral N availability low.

**Acknowledgements**

This study was partly funded by the Danish Agency for Science, Technology and Innovation. We thank Bodil Stensgaard for technical assistance, and Kristian Kristensen for statistical support.
References


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 CH$_4$ concentrations during a 72-h incubation of slurry surface crusts with initial O$_2$ concentrations of 20, 5, 3, 2, 1, and 0%, and with initial CH$_4$ concentrations of $10^2$ ppmv (a) and $10^4$ ppmv (b). Blank control contained 20% initial headspace O$_2$ 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 α = 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 $^{13}$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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface Crust</th>
<th>Slurry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td></td>
<td>μmol kg⁻¹ WW*</td>
<td>μmol kg⁻¹ WW</td>
</tr>
<tr>
<td>Cattle slurry surface crusts developed for 3 years (Duan et al., 2013)</td>
<td>116.3</td>
<td>370.9</td>
</tr>
<tr>
<td>Various cattle and swine slurries and surface crusts (Nielsen et al., 2010)</td>
<td>n.a.</td>
<td>7–3,602</td>
</tr>
<tr>
<td>Surface crusts of various dryness (Hansen et al., 2009)</td>
<td>n.a.</td>
<td>290–35,000</td>
</tr>
<tr>
<td>Various cattle and swine slurries (Sommer et al., 2007)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Surface crusts (Petersen et al., 2006)</td>
<td>1,432–54,923</td>
<td>91–16,378</td>
</tr>
<tr>
<td>Digested cattle slurry (Clemens et al., 2006)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

* WW, wet weight.

# n.a., value not reported.
Table 2: Combinations of CH$_4$, O$_2$, 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 $^{13}$C-labelled phosphate lipid fatty acids (PLFAs).

<table>
<thead>
<tr>
<th>N Species and Concentrations</th>
<th>CH$_4$: $10^2$ ppmv</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>10$^4$ ppmv</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O$_2$: 0%</td>
<td>1%</td>
<td>2%</td>
<td>3%</td>
<td>5%</td>
<td>20%</td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
<td>3%</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>3*</td>
<td>3</td>
<td>3*</td>
<td>3</td>
<td>3*</td>
<td>3</td>
<td>3*</td>
<td>3</td>
<td>3*</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>10 mM</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2*</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>2</td>
<td>2</td>
<td>2*</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>10 mM</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>2</td>
<td>2</td>
<td>2*</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>1 mM</td>
<td>2</td>
<td>2</td>
<td>2*</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: First-order rate constants (h⁻¹) of CH₄ oxidation in slurry surface crust samples under different O₂ 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 CH₄ concentration, values followed by the same letter are not significantly different at α = 0.05.

<table>
<thead>
<tr>
<th>N Species and Concentrations</th>
<th>10² ppmv CH₄</th>
<th>10⁴ ppmv CH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% O₂</td>
<td>3% O₂</td>
</tr>
<tr>
<td>None</td>
<td>0.036 (1.00) a</td>
<td>0.037 (1.00) a</td>
</tr>
<tr>
<td>NH₄⁺ 10 mM</td>
<td>0.015 (0.42) d</td>
<td>0.010 (0.27) ef</td>
</tr>
<tr>
<td>50 mM</td>
<td>0.007 (0.19) fg</td>
<td>0.005 (0.14) g</td>
</tr>
<tr>
<td>NO₃⁻ 10 mM</td>
<td>0.029 (0.81) b</td>
<td>0.022 (0.59) c</td>
</tr>
<tr>
<td>50 mM</td>
<td>0.017 (0.47) d</td>
<td>0.013 (0.35) de</td>
</tr>
<tr>
<td>NO₂⁻ 1 mM</td>
<td>0.008 (0.22) fg</td>
<td>0.008 (0.22) fg</td>
</tr>
</tbody>
</table>
Supplemental Material

Methanotrophic activity in slurry surface crusts as influenced by CH$_4$, O$_2$, 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 \textit{in situ} measurement of O$_2$ and N$_2$O 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.

<table>
<thead>
<tr>
<th>Initial State (time 0)</th>
<th>After 8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH₄ Consumed (μM)</td>
</tr>
<tr>
<td>Headspace O₂ (%)</td>
<td>Dissolved O₂ (μM)</td>
</tr>
<tr>
<td>20</td>
<td>256.0</td>
</tr>
<tr>
<td>3</td>
<td>38.4</td>
</tr>
<tr>
<td>1</td>
<td>12.8</td>
</tr>
</tbody>
</table>

- Stoichiometry of CH₄ oxidation is:
  \[ \text{CH}_4 + (2 - x)\text{O}_2 \rightarrow (1 - x)\text{CO}_2 + x\text{CH}_2\text{O} + (2 - x)\text{H}_2\text{O}, \]
  where \(x\) is the fraction of carbon that is assimilated into biomass (\(\text{CH}_2\text{O}\)) (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 \(1.75 \times 10^{-5}\) cm² sec⁻¹, respectively (Broecker and Peng, 1974). Therefore, there’s no diffusion limit of O₂ for CH₄ oxidation.