Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake

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Edited by Tim R. Moore, McGill University, Montreal, QC, Canada, and accepted by the Editorial Board November 13, 2014 (received for review June 23, 2014)

Anaerobic methane oxidation coupled to denitrification, also known as "nitrate/nitrite-dependent anaerobic methane oxidation" (n-damo), was discovered in 2006. Since then, only a few studies have identified this process and the associated microorganisms in natural environments. In aquatic sediments, the close proximity of oxygen- and nitrate-consumption zones can mask n-damo as aerobic methane oxidation. We therefore investigated the vertical distribution and the abundance of denitrifying methanotrophs related to *Candidatus* Methylomirabilis oxyfera with cultivation-independent molecular techniques in the sediments of Lake Constance. Additionally, the vertical distribution of methane oxidation and nitrate consumption zones was inferred from high-resolution microsensor profiles in undisturbed sediment cores. M. oxyfera-like bacteria were virtually absent at shallow-water sites (littoral sediment) and were very abundant at deep-water sites (profundal sediment). In profundal sediment, the vertical distribution of M. oxyfera-like bacteria showed a distinct peak in anoxic layers that coincided with the zone of methane oxidation and nitrate consumption, a strong indication for n-damo carried out by M. oxyfera-like bacteria. Both potential n-damo rates calculated from cell densities (660–4,890 μmol $CH_4 \cdot m^{-2} \cdot d^{-1}$) and actual rates calculated from microsensor profiles (31–437 μmol $CH_4 \cdot m^{-2} \cdot d^{-1}$) were sufficiently high to prevent methane release from profundal sediment solely by this process. Additionally, when nitrate was added to sediment cores exposed to anoxic conditions, the n-damo zone reestablished well below the sediment surface, completely preventing methane release from the sediment. We conclude that the previously overlooked n-damo process can be the major methane sink in stable freshwater environments if nitrate is available in anoxic zones.

n-damo | M. oxyfera-like bacteria | NC-10 bacteria | microsensor profiles | Lake Constance

Freshwater habitats contribute a major part to the global emissions of the important greenhouse gas methane (1). However, most of the methane that is formed in deeper anoxic zones of freshwater habitats (e.g., lake sediments) is oxidized before it reaches the atmosphere (2). This mitigation of freshwater methane emissions was long thought to be carried out solely by aerobic methanotrophic bacteria. After the first indications of anaerobic methane oxidation coupled to denitrification in freshwater habitats based on tracer experiments (3, 4), the discovery, enrichment, and detection of denitrifying methanotrophs (5–10) ultimately has proven that methane oxidation also takes place in anoxic zones of freshwater habitats. The process of anaerobic methane oxidation coupled to denitrification, also called "nitrate/nitrite-dependent anaerobic methane oxidation" (n-damo), is carried out by members of the candidate phylum NC10 (6) and an archaeal lineage related to the anaerobic methanotrophic (ANME) archaea (11). Of four different groups of NC10 bacteria, only group a was enriched and shown to mediate n-damo (7), but group b always was found in the same habitats (7, 12–14). The genome sequence of a group a NC10 bacterium, *Candidatus* Methylomirabilis oxyfera, led to the experimentally supported assumption that, as in aerobic methanotrophs, methane is oxidized by a methane monooxygenase [encoded by a particulate methane monooxygenase (*pmo*) gene cluster] via an intriguing intra-aerobic mechanism (15). Although 16S rRNA gene sequences and *pmoA* gene sequences that allow a functional assignment to a methanotrophic lifestyle appear in public databases, few studies have investigated the distribution and occurrence of these denitrifying methanotrophs in detail. However, a recent study claimed n-damo to be an overlooked methane sink in wetlands (13). M. oxyfera-like bacteria (group a NC10 bacteria) also were investigated in different high-nitrate environments such as wastewater treatment plants (16), a minerotrophic peatland (14), paddy soil (17), and Lake Constance (5). The Lake Constance study suggested a distinct distribution of the n-damo process and M. oxyfera-like bacteria in a nitrate-rich and seasonally stratified lake. In Lake Constance, nitrate concentrations are comparably high because of exogenous inorganic nitrogen input by rivers; nitrate concentrations increased from 30–40 μM in the early 1960s to 60–80 μM in the late 1980s and have remained constant ever since (18). Based on only one sediment core per site, M. oxyfera-like bacteria have been detected in deep-water (profundal) sediment but were absent at a shallow-water (littoral) sediment site. To estimate the contribution of n-damo in mitigating methane emissions from freshwater habitats, more comprehensive data on n-damo rates in different habitats are needed. The n-damo rates have been

Significance

Evidence whether the recently discovered denitrification-dependent methane oxidation (nitrate/nitrite-dependent anaerobic methane oxidation, n-damo) represents a major methane sink or an insignificant side aspect in the global methane cycle is scarce. High-resolution microprofiles measured in intact sediment cores close to in situ conditions, anoxic incubations of intact sediments, and quantification of the responsible microorganisms with molecular techniques proved n-damo to be the major methane sink in Lake Constance, one of the best-studied freshwater lakes. The n-damo process has long been overlooked because of the close proximity of aerobic and anaerobic activities. Our study documents that a large part of methane previously thought to be oxidized aerobically is in fact oxidized anaerobically by physiologically entirely different organisms.

Author contributions: J.S.D., P.S., and B.S. designed research; J.S.D., P.S., and J.B. performed research; B.S. analyzed data; and J.S.D., P.S., and B.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. T.R.M. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1411617111/-/DCSupplemental.

calculated based on tracer experiments (5, 13), but in situ rates based on methane and nitrate/nitrite distribution gradients have not been calculated so far (13, 14). In the special case of a minerotrophic peatland infused with nitrate-contaminated groundwater, methane apparently was oxidized completely within the anoxic zone (14). Regrettably, in high-turnover environments with high microbial activity, gradients usually are steep, and different substrates are consumed at close spatial proximity, complicating reliable measurements. In the present study, we investigated the NC10 bacterial communities in sediments of various water depths in Lake Constance. In addition, high-resolution microsensor measurements of methane, oxygen, nitrate, and nitrite gradients and anoxic incubations of intact sediment cores were conducted to assess the importance of n-damo in the mitigation of methane emissions from the microbially highly active sediments in this deep lake.

Results

Abundance and Biogeography of M. oxyfera-Like Bacteria. To assess the importance of n-damo in Lake Constance and to be able to estimate lake-wide n-damo rates, the biogeography and abundance of M. oxyfera-like bacteria were investigated. A summary of the sediments investigated can be found in Table S1. Huge numbers of NC10 group a bacterial 16S rRNA genes (10^8–10^9/cm^3) were detected in profundal sediments via quantitative PCR (qPCR). They also were detected in three of six samples from eastern Lake Constance at water depths between 10 and 15 m (10^6–10^7/cm^3) and at a 20-m-deep site near the Isle of Mainau (10^6/cm^3) (Table 1) but were not detected in shallow water sites (0–4 m water depth). *pmoA* genes were quantified successfully only from the three deep profundal samples at copy numbers (10^8–10^9/cm^3) similar to the 16S rRNA gene. In both cases, copy numbers were about one order of magnitude higher in profundal samples from southeastern Lake Constance (Table 1). Terminal fragment length polymorphism (T-RFLP) analysis of NC10 bacterial 16S rRNA genes confirmed this water depth-dependent distribution of NC10 bacteria (*SI Text*, Fig. S1, and Table S2).

Substrate Gradients and Vertical Distribution of M. oxyfera-Like Bacteria in Profundal Sediments. Oxygen was consumed rapidly in the uppermost sediment layers and penetrated to maximum depths of 4–5 mm in cores F2 and F1 and 13.5 mm in core K3. In all profundal sediment cores, methane was present at high concentrations deeper in the sediment (>20 mm), decreased toward the sediment surface, and was depleted completely at sediment depths of between 6 (F2) and 15 (K3 and F1) mm. Thus, methane and oxygen profiles did not overlap in any of the cores. Nitrate penetrated deeper into the sediment than oxygen and was depleted at a depth of 16–19 mm in all cores (Fig. 1). Nitrite was measurable in the nitrate consumption zone in only some of the sediment cores at concentrations of less than 3 μM, close to the detection limit of the microsensors (Fig. 1). These profiles clearly indicate an anoxic zone favorable for methane oxidation coupled to denitrification where denitrification and methane oxidation appear to co-occur. Local volumetric methane oxidation rates calculated from these profiles peaked between 4.8 and 44 nmol·cm^{-3}·d^{-1} at sediment depths of 1–2.5 cm. Areal methane oxidation rates extrapolated from the local rates were 80, 31, and 437 μmol·m^{-2}·d^{-1}, whereas nitrate consumption rates were 714, 498, and 1,958 μmol·m^{-2}·d^{-1} in cores K3, F1, and F2, respectively. A pronounced nitrate peak was observed in the oxygen consumption zone indicating elevated nitrification activity in sediment cores that were incubated at elevated temperatures (*ca*. 10 °C) for a few hours before measurement but was much smaller in cores measured immediately after they had reached a temperature (*ca*. 10 °C) suitable for microsensor measurements. Nitrification rates calculated from the microprofiles were 1,885 and 2,175, 710, and 3,135 μmol·m^{-2}·d^{-1} in cores K3, F1, and F2, respectively.

The vertical distribution of M. oxyfera-like bacteria corresponded well with the observed substrate gradients and the inferred zones of nitrate consumption and methane oxidation. M. oxyfera-like bacteria were virtually absent in the upper layers of all sediment cores (<1 cm), and the highest abundance of M. oxyfera-like bacteria (up to 1–10% of DAPI-stained cells; Fig. S2) was found exactly in the sediment layer that the substrate gradients indicate as the n-damo zone (Fig. 1, cores K3 and F1). In core F2, where methane concentrations were higher, M. oxyfera-like bacteria were highly abundant below a depth of 5 mm throughout the sediment layers investigated. Absolute numbers of FISH-stained M. oxyfera-like bacteria were calculated to reach up to 10^7–10^8 cells/mL. These numbers are only slightly lower than the abundance of M. oxyfera-like bacteria obtained by qPCR in cores from the same location.

Anoxic Sediment Incubations. Both oxygen and nitrate were depleted within the sediment after intact sediment cores were incubated anoxically for several days. Oxygen was introduced at low concentrations only during the measurements but never penetrated deeper than 1 or 2 mm into the sediment (Fig. 2). Before addition of nitrate to the sediment cores, methane concentrations decreased linearly from the deeper layers of the sediment up to the sediment surface, indicating diffusion into the water column without any net methane production or consumption along the diffusion path. Two days after nitrate addition, methane was depleted completely several millimeters deep in the sediment core (Fig. 2), and the concave shape of the profile clearly indicated net methane consumption at sediment depths between 5 and 25 mm. Furthermore, microsensor measurements showed that nitrate diffused into the sediment column and was consumed at the same depth as methane (Fig. 2). Because methane was consumed entirely before reaching the sediment surface in core C1 and C3, methane fluxes calculated from the linear part of the profiles into the n-damo zone represent depth-integrated methane oxidation rates that allowed n-damo

Table 1. Gene copy numbers of specific and general marker genes quantified via qPCR

Sample	M. oxyfera-like bacteria: 16S rRNA gene	M. oxyfera-like bacteria: *pmoA* gene	General bacteria: 16S rRNA gene	Aerobic methanotrophs: *pmoA* gene
LR1 (12 m)	$6.0 \pm 4.6 \times 10^5$	Nd	8.8×10^9	$3.7 \pm 1.9 \times 10^7$*
LP1 (12 m)	$7.6 \pm 3.8 \times 10^5$	Nd	1.7×10^{11}	$2.5 \pm 1.6 \times 10^9$*
LP3 (12 m)	$5.5 \pm 3.6 \times 10^6$	Nd	2.9×10^9	$1.1 \pm 0.7 \times 10^9$*
20 m	$9.0 \pm 2.4 \times 10^5$	Nd	6.1×10^9	
80 m	$1.1 \pm 0.3 \times 10^8$	$4.3 \pm 1.3 \times 10^8$	1.3×10^{10}	$2–4 \times 10^7$†
PR (82 m)	$8.1 \pm 1.6 \times 10^8$	$3.3 \pm 1.1 \times 10^9$	1.3×10^{10}	$1.0 \pm 3.3 \times 10^8$*
PP (83 m)	$7.7 \pm 4.8 \times 10^8$	$3.8 \pm 2.0 \times 10^9$	5.0×10^{10}	$7.3 \pm 2.1 \times 10^7$*

Values are given per cubic centimeter of sediment. Nd, not detected.
*Data from Deutzmann et al. (20); same DNA extracts.
†Data from Rahalkar et al. (33), same site.

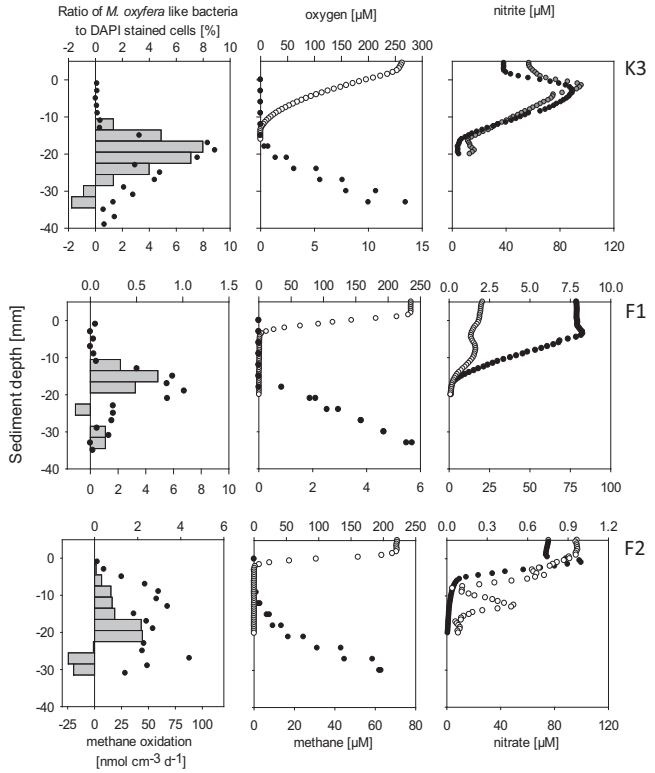

Fig. 1. Vertical distribution of *M. oxyfera*-like bacteria, substrate gradients, and calculated methane oxidation rates measured in freshly collected profundal sediment cores. (*Left*) Vertical distribution of FISH-stained *M. oxyfera*-like cells relative to total DAPI-stained cells (filled circles) and calculated methane oxidation rates obtained by analysis of the methane profiles (gray bars). (*Center*) Methane (filled circles) and oxygen (open circles) distribution within the sediment column. (*Right*) Nitrate (filled black and gray circles) and nitrite (open circles) distribution. (*Top*) Core K3. (*Middle*) Core F1. (*Bottom*) Core F2. Please note that no nitrite profile but two independent nitrate profiles were obtained for core K3.

rates to be estimated even under non–steady-state conditions. Thus, after 2 d of nitrate incubation, methane oxidation rates were 51 and 129 $\mu mol \cdot m^{-2} \cdot d^{-1}$ in cores C1 and C3, respectively. Another set of sediment cores containing higher methane concentrations showed similar profiles and even higher denitrification-dependent methane oxidation rates after incubation with 500 µM $NaNO_3$. In some cores, however, methane was largely, but not completely, oxidized within the sediment column (Fig. S3).

Discussion

Freshly collected profundal sediments of the nitrate-rich but oligotrophic Lake Constance showed that nitrate penetrates slightly more deeply into the sediment than oxygen and is depleted at the same depth as methane. This observation is a strong indication of methane oxidation coupled to denitrification. In addition, the abundance of M. oxyfera-like bacteria peaks exactly where the methane and nitrate profiles meet, indicating that indeed methane oxidation is coupled to denitrification in these sediments and that M. oxyfera-like bacteria are carrying out this process in situ.

The distribution of M. oxyfera was investigated on a lake-wide scale in Lake Constance. T-RFLP analysis revealed a dramatic shift in relative abundance from group b to group a NC10 bacteria with increasing water depth (Fig. S1 and Table S2). A similar distribution of NC10 bacteria in soils was observed recently in wetlands (13). These observations are in perfect agreement with the quantitative abundance data obtained by qPCR using the two different marker genes (16S rRNA and *pmoA*), which indicated a very similar distribution pattern. To calculate abundances from qPCR data, we assumed one copy of each gene per cell, according to their abundance in the genome sequence of M. oxyfera (15, 19). 16S rRNA gene copy numbers of denitrifying methanotrophs were unexpectedly high in profundal sediments (1–8 × 10^8/cm^3) and agreed reasonably well with the quantification of *pmoA* genes (4–38 × 10^8/cm^3). Denitrifying methanotrophs accounted for about 1–6% of total bacterial 16S rRNA genes in the 1- to 4-cm depth layer of the respective sediments, as verified by the ratio of FISH-stained M. oxyfera-like bacteria to total DAPI-stained cells. Thus, two independent methods revealed these bacteria to be one of the dominant bacterial species in the sediment of a well-investigated freshwater lake. Taken together, these results indicate that denitrifying methanotrophs far outnumber aerobic methanotrophs (*ca.* 10^7–10^8/cm^3) at undisturbed deep-water sites (Table 1). The higher abundance of denitrifying methanotrophs in the eastern part of Lake Constance coincides with the higher abundance of aerobic methanotrophs in this part of the lake (20). Both might be attributed to the higher availability of methane in this area of the lake harboring active methane seeps and to the higher input by the river Rhine of allochthonous organic material as substrate for methanogenesis (21). Although denitrifying methanotrophs obviously play a significant role at profundal sites, the ecological relevance of denitrifying methane oxidation might be negligible at shallow-water sites, even if the denitrifying methanotrophs were detectable, especially considering the ∼100 times lower cell-specific CH_4 conversion rates of the denitrifying methanotrophs (7) as compared with their aerobic counterparts (22). Other studies also found high abundances of M. oxyfera-like bacteria in Chinese wetland soils, with 1.5 × 10^6 to 3.2 × 10^7 16S rRNA gene copies/g of dry soil and the highest abundance at a soil depth of 50–60 cm (13), and in a Dutch peatland where up to 3.2 × 10^7 cells/g soil (3–8% of total bacteria) in the nitrate–methane transition zone at depth of *ca.* 1 m have been reported (14).

Although the biogeographic distribution pattern of NC10 bacterial denitrifying methanotrophs was quite obvious, the reasons determining this distribution are not. The water depth where denitrifying methanotrophs could be detected (20–25 m) coincides with the depth of thermal stratification during summertime

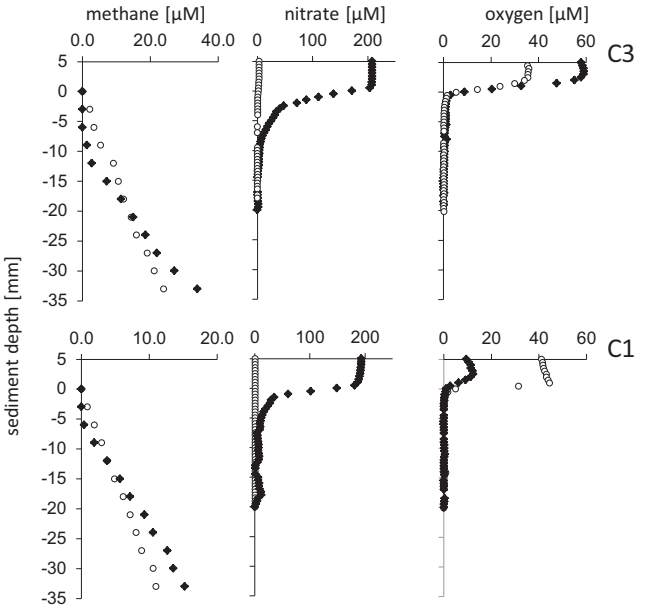

Fig. 2. Methane, nitrate, and oxygen profiles of anoxically incubated sediment cores. (*Upper*) Core C3. (*Lower*) Core C1. Open circles, profiles obtained before the addition of nitrate; filled diamonds, profiles obtained 2 d after addition of 200 µM nitrate.

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(18). During summer, nutrients in the surface water layer (epilimnion) are depleted by the growth of primary producers. In the deep water layer (hypolimnion), however, nitrate concentrations are twice as high as in the epilimnion (18) and thus might favor n-damo in the profundal zone. Additionally, the sediment at the water depth of above and around the thermocline is a very dynamic environment in Lake Constance. Disturbances such as wave action, internal waves of the thermocline, and bioturbation might introduce oxygen into the sediment periodically and physically dislocate the n-damo bacteria to unfavorable conditions. Furthermore, within the photic zone (5–15 m water depth), day–night cycles and seasonal algal or macrophyte growth change the redox gradients by introducing oxygen into the sediment by photosynthesis in the upper sediment layers or oxygenation of the rhizosphere in the deeper sediment layers. Because the denitrifying methanotrophs grow slowly even at 30 °C (doubling time, 1–2 wk at 30 °C and likely two to four times slower at 4 °C), these bacteria presumably have a limited capability to react to environmental changes or to recover from disturbances and therefore need a stable environment to reach high cell densities. This need for a stable environment also might be the reason why all enrichments so far have been obtained in continuous cultures (7, 10, 12). Because oxygen was shown to inhibit M. oxyfera (23), oxygen is a likely factor limiting the distribution of these bacteria to permanently anoxic environments, as has been suggested by other studies (13, 14). Thus, both nitrate availability and temporary oxygenation of the sediment by disturbances could be determining factors for the observed distribution of the n-damo process. In a literature review (24), very similar factors have been hypothesized to limit the occurrence of anaerobic ammonium oxidation (anammox). Because the n-damo and anammox processes and organisms are similar in many ways (e.g., nitrite dependent, very slow growing, strictly anaerobic), environmental factors limiting anammox also might apply to n-damo.

Microsensor measurements and FISH counts indicated a narrow zone of high n-damo activity in profundal sediment cores (>75 m water depth). Methane oxidation rates calculated from methane profiles with maximum values of 4.8–44 $nmol \cdot cm^{-3} \cdot d^{-1}$ were well in the range of potential n-damo rates (22 and 163 $nmol \cdot cm^{-3} \cdot d^{-1}$) based on cell counts and a cellular methane oxidation rate of 0.2 $fmol \cdot cell^{-1} \cdot d^{-1}$ (7). However, these rates are about one order of magnitude higher than the potential denitrifying methane oxidation rates determined in sediments from the same sampling location in batch experiments (5). This discrepancy likely is explained by the addition of nitrate to the incubation experiments, which means that M. oxyfera-like bacteria rely on other microorganisms to supply nitrite. The kinetics of nitrite formation after the addition of a relatively high dose of nitrate (1 mM compared with 60–80 μM in the open water) and the resulting availability of nitrite for M. oxyfera-like bacteria are unknown. Thus, the methane oxidation rates obtained after a pulse of indirectly supplied electron acceptor are unlikely to match the steady-state in situ rates. The n-damo rates measured in the present study also are about one order of magnitude higher than methane oxidation rates recently published for paddy fields, probably because turnover rates are generally lower in these fields where substrate gradients stretched over several tens of centimeters (13). However, microprofiles measured in retrieved sediment cores must be treated with caution. Oxygen profiles can shift within minutes in Lake Constance sediments because of changes in microbial activity after changing environmental conditions such as illumination (25). However, because the profundal sediment core originates from a permanently dark and cold (4–5 °C) environment, the oxygen profile presumably shifted upwards only slightly (*ca*. 10 °C) before the measurements started. This shift of the oxygen profile—and to a lesser degree of the nitrate profile—was more pronounced in cores stored for a few hours before the measurements started (cores K3 and F2), thus corroborating this hypothesis. In addition, a pronounced nitrification peak was observed in the stored cores but was less significant in the most freshly measured core (F1), suggesting that under in situ conditions nitrification might play only a minor role in supplying nitrate to anoxic sediment layers as compared with the nitrate flux directly from the water column. Despite these possible shifts in oxygen and nitrate profiles, the consumption zones of nitrate and methane still overlapped, and the co-occurrence of M. oxyfera-like bacteria, methane oxidation, and nitrate consumption at the same depth is strongly indicative of n-damo. Nevertheless, because of the close proximity and possibly slightly altered distribution of the oxygen and nitrate profiles, additional evidence for the importance of n-damo was needed.

This evidence was gained from nitrate amendments to anoxic incubations of profundal sediment cores. In these sediment cores, methane was oxidized largely or even completely in the absence of oxygen only when nitrate became available. This observation proves that the indigenous microbial community is able to oxidize all the methane produced deeper in the sediment core and to mitigate the release of methane to the overlying water column. Methane oxidation rates reached ∼50–130 $\mu mol \cdot m^{-2} \cdot d^{-1}$, in good agreement with the rates measured in freshly collected sediment cores. A similar study using microsensor as well as rate measurements in 16-mo-incubated freshwater sediments with high methane production rates of 2,000–2,100 $\mu mol \cdot m^{-2} \cdot d^{-1}$ found high n-damo rates of 100 $\mu mol \cdot m^{-2} \cdot d^{-1}$ and 2,300 $\mu mol \cdot m^{-2} \cdot d^{-1}$ in control and nitrate-amended (1.0–3.5 mM) treatments, respectively (26). In that study, n-damo accounted for 11% and 47% of the total (aerobic + anaerobic) measured methane oxidation rate in tracer experiments and for 4% and 140% of the methane flux from below in control and nitrate-amended treatments, respectively, indicating nitrate limitation of the n-damo process in this sediment. In a peatland, nitrate concentrations of 400–700 μM in the surface layer also were sufficient to oxidize methane completely at fluxes of *ca*. 150–250 $\mu mol \cdot m^{-2} \cdot d^{-1}$ by n-damo, as estimated from published CH_4/NO_3^- gradients (14).

In all these studies, relatively high nitrate concentrations in the water column (or soil surface) resulted in nitrate fluxes high enough to achieve complete anaerobic oxidation of methane. In oligotrophic environments with typically lower methane fluxes, nitrate concentrations in the range of ten to a few hundred micromolars are sufficient to achieve complete methane oxidation by n-damo. Another common factor in these studies was that n-damo was observed only in habitats where environmental conditions presumably are very stable and no periodical disturbances occur that might expose n-damo organisms to oxygen. Thus, n-damo might be widespread in lakes and other stable freshwater environments where nitrate concentrations are low and methane fluxes are high but may constitute a major (and measurable) methane sink only where nitrate concentrations are sufficiently high to balance methane fluxes. However, to our knowledge, no reports of the abundance or distribution of M. oxyfera-like bacteria or n-damo rates are available for freshwater lakes except for Lake Biwa, which has a relatively low abundance of M. oxyfera-like bacteria (maximum, $10^6/cm^3$) and low nitrate concentrations (*ca*. 20 μM) to balance a high methane flux (*ca*. 2.5–3 $mmol \cdot m^{-2} \cdot d^{-1}$) (27). Thus, additional research is needed to estimate accurately the importance of n-damo on methane oxidation in lakes on a global scale.

Despite the evidence provided in this study that anaerobic methane oxidation is the dominant methane sink in Lake Constance, the co-occurrence of aerobic and anaerobic methanotrophs in profundal sediments is puzzling. However, it is known that aerobic methanotrophs can be dormant for extended periods of time but recover quickly and resume methane oxidation activity (28) and also may be able to survive prolonged starvation by fermentation (29). In addition, methane fluxes may vary because of variable methane production rates [caused, for example, by seasonally sedimenting algal blooms (30)], or occasional

bioturbation might mix methane and oxygen profiles. Combined with the low temperatures and high doubling times of the M. oxyfera-like bacteria, anaerobic methane oxidation might be sufficient to oxidize average methane fluxes completely in profundal sediments, but aerobic methanotrophs might mitigate methane emissions from occasionally high methane fluxes through their ability to react and divide quickly upon changing environmental conditions.

Of the total surface area of Lake Constance (473 km^2), n-damo likely is a significant methane sink in the 402 km^2 that are >15 m deep and could mitigate methane emissions mostly or even completely in the 274 km^2 of the lake sediment that are >75 m deep. These areas correspond to estimated n-damo rates of 40–400 tons of CH_4 per annum in the deep part (>75 m) of Lake Constance alone, not including the present but not yet quantified rates at water depths between 15 and 75 m. Thus, even in a well-studied environment such as Lake Constance, where methane oxidation has been investigated for decades (31–33), this previously unidentified process can be the major methane sink. However, this process has been overlooked because it was masked as aerobic methane oxidation by the close proximity of oxygen and nitrate consumption zones in the sediment. This flawed interpretation of sedimentary methane cycling might be a widespread phenomenon: High local n-damo rates might be misinterpreted as aerobic methane oxidation because the spatial resolution of sampling is not high enough and nitrate and nitrite usually are consumed very quickly in anoxic incubation experiments.

In enrichment cultures of n-damo organisms, nitrite has been reported to be the preferred or only electron acceptor for methane oxidation. Thus, nitrite for n-damo is either supplied by commensal microorganisms or generated by the n-damo bacteria themselves by oxidation of electron donors other than methane (14). Nitrate profiles measured in freshly collected profundal sediment cores revealed nitrate consumption rates 5–10 times higher than methane oxidation rates, indicating that n-damo consumes only a fraction of this electron acceptor and that other members of the community participate in nitrate reduction and likely supply the nitrite needed by M. oxyfera-like bacteria. In line with this notion, some microprofiles revealed small concentration peaks of nitrite in the nitrate consumption zone (Fig. 1), implying that nitrite is supplied by members of the denitrifying community. Recently, a group of ANME-related archaea has been shown to couple methane oxidation to nitrate reduction to nitrite. Thus, these archaeal methanotrophs not only could supply nitrite to M. oxyfera-like bacteria but also could participate in anaerobic methane oxidation. The importance of these denitrifying ANME archaea in methane oxidation in Lake Constance merits further research.

This study provides concurrent evidence that n-damo can be a dominant methane sink in deep lakes but might be masked and misinterpreted as aerobic methane oxidation because of the close spatial proximity of oxygen and nitrate/nitrite profiles. Nonetheless, very high abundances of M. oxyfera-like bacteria detected by qPCR and FISH combined with high-resolution microsensor measurements and incubation experiments clearly show that methane oxidation coupled to denitrification not only is an overlooked process (13) but also can be the dominant methane sink in stable, nitrate-rich environments.

Materials and Methods

Sampling. All sediment cores were sampled as described previously (5), and a comprehensive list of all samples can be found in Table S1. Only few tubificid worms (5–10) were observed in the sediment cores as macrozoobenthos potentially involved in bioturbation. For molecular analysis of the NC10 bacterial distribution (qPCR and T-RFLP), the uppermost 1 cm of sediment was discarded to remove macrozoobenthos and macrophytes to minimize the content of eukaryotic DNA in the samples. The sediment layer at a depth of 1–4 cm was homogenized and used for further molecular analysis. Additionally, samples obtained from methane seeps in a previous study were analyzed (20).

For microsensor measurements, for depth distribution of M. oxyfera-like bacteria in the sediment cores (FISH), and for anoxic incubation experiments, only intact and freshly collected profundal sediment cores (*ca*. 80 m water depth) were used.

Molecular Methods. DNA of the samples obtained in this study was extracted in duplicate as described previously (20). qPCR was performed using 10 ng template DNA, SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) on an ABI 7500 fast machine. NC10 bacterial 16S rRNA genes were quantified using the primer pair qP2F–qP2R (7), and partial *pmoA* genes were amplified using the primer pair CMO182–CMO568 (9). A plasmid (PCR2.1; Invitrogen) containing a single copy of the target gene was serially diluted 1:10 to cover the range of 10^1–10^7 molecules per PCR mixture, and each dilution was used as the standard in triplicate. Two replicates of each duplicate DNA extraction of freshly collected samples in this study or three replicates of previously collected samples were analyzed. PCR products were visualized via gel electrophoresis and ethidium bromide staining to exclude formation of unspecific PCR products. Gene copy numbers were calculated with the LinRegPCR program (34), setting individual amplification efficiencies for the different samples but using one common threshold, and Microsoft Excel. Although the plasmids used as standards were detected successfully at 10^3 copies per reaction for both genes, formation of unspecific products during the *pmoA* qPCRs prohibited the detection of NC10-related *pmoA* genes at concentrations of less than 10^5 copies per reaction (~10^8 copies/cm^3) from environmental samples. The detection limit of the NC10-16S qPCR was less than 10^3 per reaction (~10^6/cm^3) for environmental samples. Cell numbers were calculated using the obtained gene copy numbers, the amount of sediment used for DNA extraction, and the amount of DNA extracted from sediment. One copy of the *pmoA* and one copy of the 16S rRNA gene were assumed to be present per cell (15). A short discussion about the qPCR method can be found in *SI Materials and Methods*.

Gradient Measurements. Methane gradients were measured with a diffusion-based methane sensor as described elsewhere (32). Oxygen gradients were measured with a Clark-type microelectrode (Ox 50 or Ox100; Unisense). Nitrate and nitrite gradients were measured with NO_x and nitrite microbiosensors, respectively (35, 36), that were constructed at the Max Planck Institute for Marine Microbiology. The NO_x and nitrite sensors were calibrated before and after profiling in tap water spiked with aliquots of $NaNO_3$ and $NaNO_2$ stock solutions (10 mmol/L each) to nominal concentrations of 0–100 μmol/L NO_3 and 0–20 μmol/L NO_2. The calibration curves were corrected for the background concentrations of nitrate and nitrite in the tap water as determined with the VCl_3 reduction method (37). The NO_x sensor measures the sum of nitrate, nitrite, and nitrous oxide, but here the NO_x profiles are reported as nitrate profiles because nitrite and nitrous oxide concentrations in the sediment were very low. Similarly, the profiles measured with the nitrite sensor (which measures the sum of nitrite and nitrous oxide) are reported as nitrite profiles.

Microsensor profiles were measured in freshly collected sediment cores within 8 h of sampling to minimize shifts in concentration gradients caused by artificial incubation conditions. Because of the temperature requirements of the biosensors, all microprofiles were measured at *ca*. 10 °C. The first sediment core of each series was measured within about 2 h after sampling. Replicate methane profiles were measured with a previously described measuring set-up (32) at randomly chosen positions in each sediment core down to 30–40 mm below the sediment surface in increments of 3 mm. Oxygen, NO_x, and nitrite microsensors were used simultaneously as in a previously described measuring set-up (38, 39). Replicate oxygen, NO_x, and nitrite profiles were recorded at randomly chosen positions in each sediment core from 5 mm above to 20 mm below the sediment surface in increments of 0.5 mm.

The curve of steady-state methane, NO_x, and nitrite concentration profiles was used to derive net rates of local production and consumption by diffusion-reaction modeling (40) in spreadsheet calculations as described in detail elsewhere (41). The twofold derivation of the concentration profiles in this calculation procedure leads to a slight vertical spreading of production and consumption zones in the sediment column. The diffusion coefficients of methane, NO_x, and nitrite at 10 °C in water (D_0) were taken as 1.32×10^{-5} cm^2/s, 1.23×10^{-5} cm^2/s, and 1.24×10^{-5} cm^2/s, respectively (42, 43). The effective diffusion coefficients in the sediment (D_s) were calculated as $D_s = D_0 \times \text{Porosity}^2$, where porosity corresponds to the volumetric water content of the lake sediment, which was 0.9. Depth-integrated rates of methane and NO_x consumption were calculated by summing up the local consumption rates multiplied by the thickness of the respective depth interval of the concentration profiles. Fluxes of methane into the water column or into the

zone of anaerobic methane oxidation were calculated from the linear parts of the concentration profiles using Fick's first law of diffusion.

FISH. To investigate the depth distribution of M. oxyfera-like bacteria, subcores were taken after the gradient measurements using cut-off 60-mL syringes (2.6 cm diameter) that were sealed by inserting another plunger from the bottom side. Sediment then was sliced into 2-mm-thick slices by pushing the lower plunger slowly upwards and using a device previously designed to cut sediment (44) to cut and transfer the sediment onto cover glasses (22 × 50 mm, 0.16–0.19 mm thickness) for microscopy. Sediment then was transferred into 2-mL reaction tubes. Cells were extracted from sediment as described previously (33), and FISH was performed as described elsewhere (6) but using only a Cy3-labeled mixture (1:1) of two probes targeting M. oxyfera-like bacteria: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 (10). Cells were counterstained with 20 μL DAPI (1 μg/mL) for 2 min. In total, at least five large (5 × 0.015 mm^2) squares and at least 5 × 100 DAPI-stained cells were counted.

Anoxic Sediment Incubation Experiments. Intact sediment cores were incubated at 18 °C. Sediment cores were sealed with a plastic lid fitted with a rubber stopper and were incubated anoxically by bubbling N_2 continuously through the overlying water column for at least 4 d before 500 μM (core A, C, and D) or 200 μM (cores C1 and C3) $NaNO_3$ was added. Addition of nitrate at these high concentrations (compared with 60–80 μM in ambient profundal lake water) was necessary to achieve comparable penetration depths of nitrate into the sediment. Methane (all cores) and NO_x (cores C1 and C3) profiles were measured before and after nitrate addition at the intervals indicated in *Results*. Methane profiles were measured under microoxic conditions by opening the core and quickly covering the sediment core with a Styrofoam disk with holes fitting the necessary measurement equipment; a stream of N_2 was used to flush the headspace continuously. To minimize oxygen contamination, NOx profiles were measured while N_2 was bubbled through the overlying water column of the sediment cores. Oxygen gradients were measured during those measurements to estimate the influence of introduced oxygen on the observed gradients.

ACKNOWLEDGMENTS. We thank Dirk de Beer and the technicians of the Microsensor Group at the Max Planck Institute for Marine Microbiology for supplying microsensors and measuring equipment, Alfred Sulger and the crew of the *RV Robert Lauterborn* for taking the profundal sediment samples, and Martin Wessels and the Institute for Lake Research for supplying morphological information on Lake Constance. This study received financial support (to B.S.) from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg Project Schi180/14 and research support from the University of Konstanz.

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