



Response of the Anaerobic Methanotroph “*Candidatus Methanoperedens nitroreducens*” to Oxygen Stress

Simon Guerrero-Cruz,^a Geert Cremers,^a Theo A. van Alen,^a Huub J. M. Op den Camp,^a  Mike S. M. Jetten,^{a,b,c} Olivia Rasigraf,^a Annika Vaksmaa^{a,d}

^aDepartment of Microbiology, IWWR, Radboud University Nijmegen, Nijmegen, the Netherlands

^bDepartment of Biotechnology, Delft University of Technology, Delft, the Netherlands

^cSoehngen Institute of Anaerobic Microbiology, Nijmegen, the Netherlands

^dRoyal Netherlands Institute for Sea Research, Texel, the Netherlands

ABSTRACT “*Candidatus Methanoperedens nitroreducens*” is an archaeon that couples the anaerobic oxidation of methane to nitrate reduction. In natural and man-made ecosystems, this archaeon is often found at oxic-anoxic interfaces where nitrate, the product of aerobic nitrification, cooccurs with methane produced by methanogens. As such, populations of “*Ca. Methanoperedens nitroreducens*” could be prone to regular oxygen exposure. Here, we investigated the effect of 5% (vol/vol) oxygen exposure in batch activity assays on a “*Ca. Methanoperedens nitroreducens*” culture, enriched from an Italian paddy field. Metagenome sequencing of the DNA extracted from the enrichment culture revealed that 83% of 16S rRNA gene reads were assigned to a novel strain, “*Candidatus Methanoperedens nitroreducens Verserenetto*.” RNA was extracted, and metatranscriptome sequencing upon oxygen exposure revealed that the active community changed, most notably in the appearance of aerobic methanotrophs. The gene expression of “*Ca. Methanoperedens nitroreducens*” revealed that the key genes encoding enzymes of the methane oxidation and nitrate reduction pathways were downregulated. In contrast to this, we identified upregulation of glutaredoxin, thioredoxin family/like proteins, rubrerythrins, peroxiredoxins, peroxidase, alkyl hydroperoxidase, type A flavoproteins, FeS cluster assembly protein, and cysteine desulfurases, indicating the genomic potential of “*Ca. Methanoperedens nitroreducens Verserenetto*” to counteract the oxidative damage and adapt in environments where they might be exposed to regular oxygen intrusion.

IMPORTANCE “*Candidatus Methanoperedens nitroreducens*” is an anaerobic archaeon which couples the reduction of nitrate to the oxidation of methane. This microorganism is present in a wide range of aquatic environments and man-made ecosystems, such as paddy fields and wastewater treatment systems. In such environments, these archaea may experience regular oxygen exposure. However, “*Ca. Methanoperedens nitroreducens*” is able to thrive under such conditions and could be applied for the simultaneous removal of dissolved methane and nitrogenous pollutants in oxygen-limited systems. To understand what machinery “*Ca. Methanoperedens nitroreducens*” possesses to counteract the oxidative stress and survive, we characterized the response to oxygen exposure using a multi-omics approach.

KEYWORDS “*Candidatus Methanoperedens nitroreducens*”, anaerobic oxidation of methane, NC10 phylum bacteria, oxidative stress

Nitrite- or nitrate-dependent anaerobic oxidation of methane (nitrite-AOM or nitrate-AOM, commonly referred to as N-AOM) is a microbial process performed by two groups of microorganisms. Anaerobic oxidation of methane coupled to nitrite

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Address correspondence to Annika Vaksmaa, avaksmaa@science.ru.nl.

reduction is catalyzed by "*Candidatus Methyloirabilis oxyfera*"-like bacteria (members of the NC10 phylum bacteria) (1–3), whereas AOM coupled to nitrate reduction is performed by "*Candidatus Methanoperedens nitroreducens*" archaea from the anaerobic methanotroph ANME-2d clade (4). In nature and man-made ecosystems, these organisms often cooccur in close proximity, where "*Ca. Methanoperedens nitroreducens*" supplies nitrite as an intermediate, which is used by "*Candidatus Methyloirabilis*" bacteria to perform nitrite-AOM (5, 6). "*Candidatus Methyloirabilis*" bacteria employ a unique intra-aerobic pathway with particulate methane monooxygenase (pMMO) and a putative nitric oxide dismutase (NOD) as the key enzymes (1), a feature that is so far exclusive to the genus "*Candidatus Methyloirabilis*" (3). "*Ca. Methanoperedens nitroreducens*" carries out reverse methanogenesis and harbors the key enzyme methyl-coenzyme M reductase (MCR), which catalyzes either the last step of methane production in methanogens or the first step of methane oxidation in anaerobic methanotrophic archaea (4). Together, "*Ca. Methanoperedens nitroreducens*" and NC10 phylum bacteria contribute to the mitigation of methane, nitrite, and nitrate in diverse, mainly freshwater environments, ranging from peatlands (7), freshwater lake sediments (8), and paddy field soils (9–11) to wastewater treatment systems (12, 13). The availability of general and specific molecular methods to identify and detect "*Ca. Methanoperedens nitroreducens*" (14, 15) is increasing the environmental detection of these organisms. Due to the very long (6- to 18-month) incubation times needed, only a limited number of successful "*Ca. Methanoperedens nitroreducens*" enrichment cultures have been reported since the original enrichment described in 2006 (4–6, 16, 17). Some studies added ammonium as a nitrogen source to the medium to stimulate the activity of anammox bacteria for nitrite removal (4). In the absence of ammonium, "*Candidatus Methyloirabilis*" bacteria are generally coenriched with "*Ca. Methanoperedens nitroreducens*" (5, 6). All documented AOM enrichments have two strategies in common, namely, supply of methane as the electron donor and supply of substrate-limited nitrate and/or nitrite as the electron acceptor. The majority of studies reported 20% to 78% enrichment of "*Ca. Methanoperedens nitroreducens*" in bioreactors, and thus physiological studies of it independent from those of "*Candidatus Methyloirabilis*" or anammox bacteria are so far not possible.

In nature, these AOM microorganisms thrive where methane, nitrate, nitrite, and ammonium (as a natural precursor for nitrite and nitrate) cooccur in the same environment. Methane and ammonium are the predominant products of anaerobic decomposition of biomass. Nitrite and nitrate are formed by aerobic ammonia oxidation when oxygen is available in upper soil and sediment layers or from partial nitrate reduction when a limited amount of (organic) electron donor is available for denitrification (18). Directly after potential electron acceptors become depleted, methanogens commence producing methane, which usually occurs in the deeper anoxic soil layers. The produced methane diffuses into the upper sediment layers; however, only a fraction reaches the atmosphere, since anaerobic, and ultimately aerobic, methanotrophs act as a methane biofilter and convert methane to carbon dioxide (19–21).

In natural environments, aerobic methanotrophic bacteria (MOB) have been found in close proximity to N-AOM organisms, since methane is a common substrate for both guilds. In ecosystems such as paddy fields, where oxic and anoxic compartments form a complex patchwork, both MOB and N-AOM have been found to be present in bulk soil, as well as in the rhizosphere (10, 11). A similar situation could be encountered in wastewater sludge aggregates with oxic outer layers and inner anoxic zones (22). In wastewater plants (WWTP), which are man-made aquatic ecosystems, pollutants are converted to less harmful compounds by microorganisms. Here, organic matter is either converted into carbon dioxide in an activated sludge process or to methane by methanogens in anaerobic digesters. The produced methane can then be used as biogas (23). In view of the current evidence on the importance of the N-AOM process in multiple natural environments (24), its potential application in existing wastewater treatment configurations has been recently discussed (25, 26). The possibilities, opportunities, and challenges of this implementation have been reviewed in depth (27, 28).

Since N-AOM organisms thrive in close proximity to oxic zones, regular exposure to oxygen can be expected due to environmental fluctuations in both natural and man-made ecosystems (29). Thus, handling and/or detoxification of oxygen is one of the challenges for the application of these anaerobic microorganisms in WWTP.

The detrimental effects of trace and low oxygen concentrations on "*Ca. Methylo-mirabilis oxyfera*" have been previously investigated (30, 31). However, so far, no report is available for the effect of oxygen on "*Ca. Methanoperedens*" species. Numerous studies have focused on the oxygen tolerance of methanogens (see below), which are phylogenetically the closest relatives to "*Ca. Methanoperedens nitroreducens*."

Both "*Ca. Methanoperedens nitroreducens*" and methanogens are traditionally considered to be strict anaerobes due to the negative effect of oxygen on enzymes of the core methane metabolism; specifically, the MCR complex becomes inactivated upon exposure to oxygen (32, 33). It has been observed that methane production is halted upon oxygen exposure in WWTP granular sludge and in anaerobic digesters (34, 35) and paddy field soil slurries (36, 37). The tolerance for oxygen of pure cultures of methanogens appears to be species dependent. The permafrost methanogen *Methanosarcina* SMA-21 remained active after 1 to 3 h of exposure to full oxic conditions, whereas after 24 h and 72 h the viability of cells declined by 85% and 90%, respectively (38). Another methanogen investigated, *Methanobacterium* MC-20, lost viability after 24 h of oxygen exposure (38). *Methanococcus voltae* and *Methanococcus vannielii* lost 99% of viability after 10 h of exposure to full oxic conditions (39).

Although methanogens and anaerobic methanotrophs are regarded as strict anaerobes and exhibit high sensitivity to oxygen, there is emerging evidence that these organisms thrive at suboxic interfaces or even in regularly aerated environments, such as forest soils, termite guts, agricultural soils, savanna and desert soils (40), meadows, wadi, and natural fields (41), cold desert soils (42), as well as paddy field soil and rhizosphere, which undergo phases of tilling and dry-wet cycles (11, 43). In oxygenated wetland soils, "*Candidatus Methanotherix paradoxum*" has been demonstrated to be abundant, active, and contributing significantly to methane production (44). Other methanogens start to produce methane again once anoxic conditions have been reestablished after oxygen exposure (40, 41). To survive the exposure to oxygen, anaerobes need to counteract the damage caused by oxygen and reactive oxygen species (ROS) by defense and repair mechanisms (45). Systems to counteract oxidative stress that have been characterized in detail include those of superoxide dismutase (SOD) and catalase (KAT) (46, 47). In the methanogens *Methanosarcina barkeri*, *Methanobrevibacter arboriphilus*, and *Methanocellales*, the catalase gene (KatE) has been identified and active transcription has been demonstrated for aerotolerant *Methanosarcina* and *Methanocella* species (48, 49). SOD has been found in *Methanosarcina barkeri* (50), *Methanocella paludicola* (formerly called the rice cluster I group) (49) as well as in *Methanobacterium thermoautotrophicum* (51). Anaerobes are known to utilize NADPH-rubredoxin oxidoreductase, rubrerythrin, and superoxide reductase to counteract the ROS, and these enzymatic reactions do not produce additional reactive oxygen species (46). The ability of methanogens to counteract oxidative damage and tolerate oxic conditions is most likely due to these defense mechanisms (49), permitting them to thrive in the rhizosphere despite oxygen release from roots.

In the current study, we performed metagenome sequencing of N-AOM enrichment culture dominated (83%) by "*Ca. Methanoperedens nitroreducens*" and reported a high-quality draft genome of this new "*Ca. Methanoperedens nitroreducens* Verse-*renetto*" strain. Furthermore, we investigated the change in the active microbial community upon oxygen exposure and characterized the metabolic response by activity assays, as well as addressed the alterations in expression of genes encoding key metabolic and oxidative stress response enzymes of "*Ca. Methanoperedens nitroreducens*." So far, no other reports have documented the response of anaerobic "*Ca. Methanoperedens nitroreducens*" archaea to oxygen exposure.

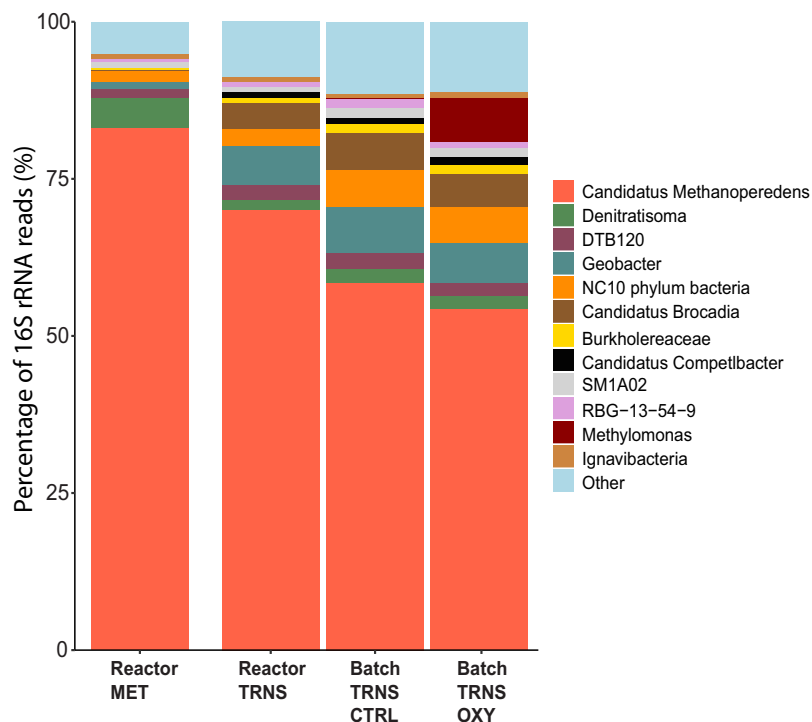


FIG 1 Taxonomic 16S rRNA gene read distribution obtained from the metagenome (Reactor MET) and metatranscriptome (Reactor TRNS) sequencing of the bioreactor, and metatranscriptome sequencing of batch incubations supplied with 1 mM nitrate in the medium and 10% methane in the headspace (Batch TRNS CTRL) or 1 mM nitrate, 10% methane, and 5% oxygen in the headspace (Batch TRNS OXY). Each column bar for transcriptomes represents the average of three replicates.

RESULTS

Community composition based on metagenome and metatranscriptome sequencing. Both DNA and RNA were extracted from the community after 8 months of stable operation and sequenced by MiSeq technology. Phylogenetic analysis of the 16S rRNA gene reads retrieved from metagenome sequencing showed that 83% of the reads were assigned to *Archaea* and 17% to *Bacteria*. All obtained archaeal reads belonged to “*Ca. Methanoperedens nitroreducens*,” the dominant microorganism in this enrichment culture. In the bacterial community, 4.9% of the reads were assigned to the genus *Denitratisoma*, followed by NC10 phylum bacteria (1.9%), *Deltaproteobacteria* environmental group *DTB120* (1.5%), and *Geobacter* (1%). The 16S rRNA gene distribution of the reactor microbial community is depicted in Fig. 1 (Reactor MET).

Metatranscriptome sequencing of the enrichment culture, control incubations, and oxygen-amended incubations confirmed that the only archaeon present was “*Ca. Methanoperedens nitroreducens*.” The metatranscriptome of the enrichment culture (Fig. 1, Reactor TRNS) indicated that the active community comprised 70% of “*Candidatus Methanoperedens nitroreducens*,” followed by bacteria from the genera *Geobacter* (6.4%), “*Candidatus Brocadia*” (4.1%), NC10 phylum bacteria (2.7%), *DTB120* (2.4%), and *Denitratisoma* (1.5%). A similar distribution pattern was observed for the 16S rRNA genes in the metatranscriptomes of the batch incubations with (Fig. 1, Batch TRNS OXY) and without (Fig. 1, Batch TRNS CTRL) addition of oxygen after 24 h. In the control batch incubation with nitrate and methane, 58.4% of the 16S rRNA reads were assigned to “*Candidatus Methanoperedens nitroreducens*,” compared to 54.3% in the incubations exposed to oxygen. The transcriptomes of the reactor biomass and anoxic batch incubations with only nitrate and methane contained less than 0.1% of 16S rRNA reads that could be assigned to the genus *Methylomonas*, an aerobic type I methane-oxidizing bacterium. In incubations with oxygen, however, 7% of the total 16S rRNA gene reads could be assigned to *Methylomonas* sp. after 24 h of exposure.

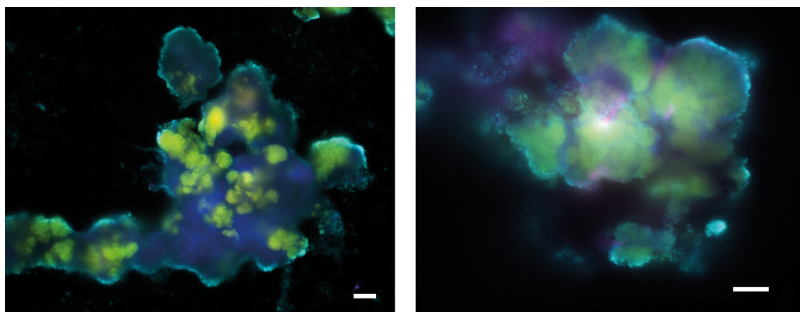


FIG 2 Fluorescent micrographs of the granular biomass of the "*Ca. Methanoperedens nitroreducens*" enrichment culture. "*Ca. Methanoperedens nitroreducens*" archaeal clusters are shown in green (combination of specific "*Ca. Methanoperedens nitroreducens*" probe DARCH 641-CY3 and general archaeal probe ARCH 915-Fluos) and NC10 phylum bacteria in purple (combination of DBACT 193-CY3 and general bacterial probe EUBMIX-CY5). The "*Ca. Methanoperedens nitroreducens*" clusters occur as a core surrounded by a dense capsule consisting of a mix of EPS stained by 4',6-diamidino-2-phenylindole (DAPI) in cyan and a side population of bacteria in blue (EUB MIX-CY5). Bar, 20 μ m.

Fluorescence *in situ* hybridization. To verify the community composition, fluorescence *in situ* hybridization with specific probes was periodically carried out. "*Ca. Methanoperedens nitroreducens*" archaea were clearly labeled in a sample of granular biomass from the bioreactor enrichment culture. The typical granule morphology was characterized by a dense agglomeration encapsulated with a thick matrix of exopolymeric substances (EPS). In a representative granule (Fig. 2), the capsule of EPS emitted the typical fluorescent signature around the macrostructure of the granule. This forms a diffuse layer on the specimen in two-dimensional space, making it difficult to achieve a higher-resolution image. Regarding the microbial community composition of the biomass, the image results confirmed the 16S rRNA gene analysis. The granular structure of the biomass was dominated by "*Ca. Methanoperedens nitroreducens*," showing a typical cauliflower configuration of archaeal aggregates (depicted in green). This has been observed previously on a related archaeon (5). NC10 phylum bacteria (depicted in purple) formed just a small fraction of total bacteria (depicted in blue) (Fig. 2).

Genome sequence of "*Candidatus Methanoperedens nitroreducens*" strain Verserenetto. Illumina sequencing of the total metagenome resulted in 7,950,503 reads, with an average length of 275.8 bp after quality trimming. After *de novo* assembly and binning, the assembled metagenome sequences assigned to "*Candidatus Methanoperedens nitroreducens*" resulted in a high-quality draft genome of 67 contigs, with an N_{50} value of 92,594 bp. The genome size was 3.51 Mb and the completeness was 99.4%, with 2.6% contamination as assessed with CheckM. The overall GC content of the genome was 40.8%, and it contains 3,829 assigned open reading frames. The genome contained all key functional genes for the reverse methanogenesis pathway and nitrate reduction. Similarly to "*Candidatus Methanoperedens nitroreducens* BLZ2," we found two copies of the *narG* gene (52). Phylogenetic analysis showed that the 16S rRNA gene sequence of our "*Ca. Methanoperedens nitroreducens*" clustered with that of the previously described "*Candidatus Methanoperedens nitroreducens* BLZ1" (99% identity) (Fig. 3). The diagnostic *mcrA* gene was 95% similar to that of "*Candidatus Methanoperedens nitroreducens* BLZ2" (GenPept accession number [WP_097300250](#)) and 92% similar to that of "*Candidatus Methanoperedens nitroreducens* ANME2D" (GenPept accession number [WP_048089615](#)) at the protein level. The strain described in this study was named "*Candidatus Methanoperedens nitroreducens* Verserenetto."

Batch activity assays with and without the amendment of oxygen. To study the effect of oxygen, biomass from the reactor was transferred to serum bottles. The oxygen levels in all incubations amended with oxygen dropped from 5% in the headspace at the start to 3.6%, on average, after 12 h of incubation. After 18 h, the readdition of oxygen to 4.4% of headspace concentration resulted in faster oxygen consumption, with residual 3.1%

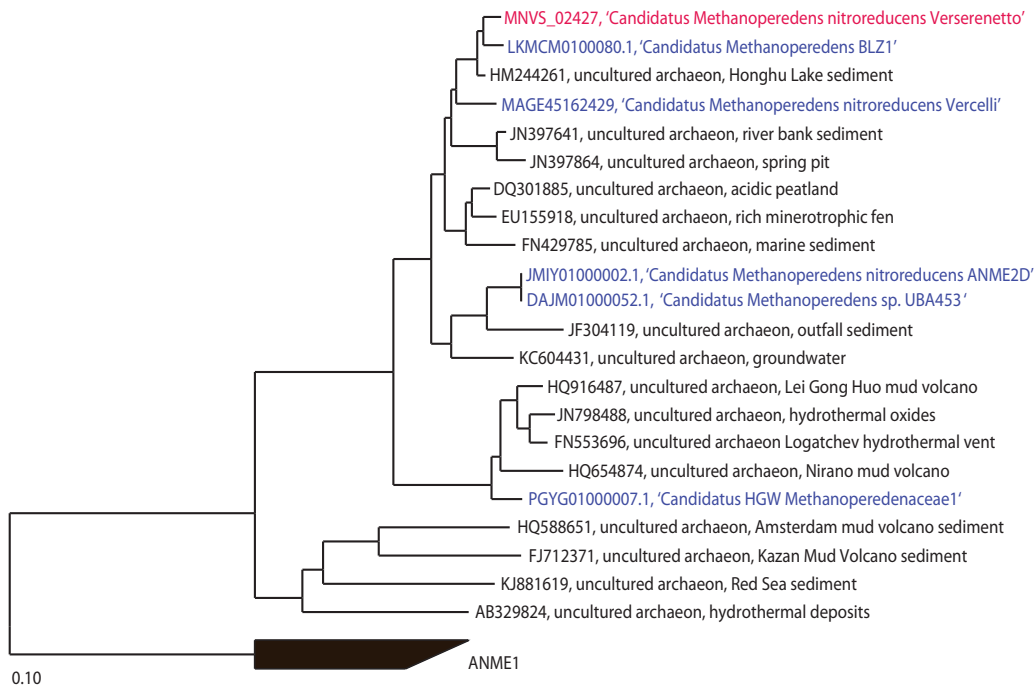


FIG 3 Phylogenetic tree illustrating the relationship of the assembled 16S rRNA gene of "*Ca. Methanoperedens nitroreducens Verserenetto*" to closely related sequences. The phylogenetic tree was constructed using the ARB package with the neighbor-joining method. The phylogenetic tree was rooted to the ANME1 cluster. The scale bar represents the difference of 0.1 substitutions per site.

oxygen after 21 h and 0.4% after 24 h exposure. The methane conversion in the control (10% methane and 1 mM NaNO₃) incubations started immediately, whereas in the oxic batch incubations, a 6-h lag phase was observed. Over the first 6 h of incubations the respective rates for methane oxidation were $4.9 \pm 0.2 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ (CRTL), $2.6 \pm 0.2 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ (10% methane, 1 mM NaNO₃, and 5% oxygen) and $3.4 \pm 0.9 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ (10% methane and 5% oxygen).

Oxic incubations showed on average a higher methane-oxidizing activity over a 24-h period in comparison to that of the controls (Table 1). The measured protein concentration of the enrichment samples was $1.4 \pm 0.1 \text{ mg} \cdot \text{ml}^{-1}$.

Response of the methanotrophic bacterial community to oxygen exposure.

After the observation that oxygen addition stimulated methane consumption, we also analyzed the 16S rRNA and *pmoA* genes present in the metatranscriptome samples, with an emphasis on the relative abundance of methanotrophic bacteria. Both intra-aerobic methane-oxidizing NC10 phylum bacteria and aerobic methanotrophs utilize a membrane-bound methane monooxygenase (pMMO) for methane oxidation. The presence of *pmoA* (encoding one of the subunits of pMMO) gene content and taxonomic distribution was analyzed (Fig. 4). In the metagenome, $5 \times 10^{-4}\%$ of all reads were assigned as *pmoA*, all encoding PmoA of NC10 phylum bacteria. In the reactor transcriptome, $7 \times 10^{-4}\%$ of all reads were assigned to *pmoA* of the NC10 phylum bacteria and $7 \times 10^{-5}\%$ were assigned to *Methylomonas* sp. *pmoA*. This was comparable with the control batch incubations with nitrate and methane. However, in batch

TABLE 1 Average methane oxidation rates of batch incubations^a

Batch incubation	Activity (nmol · h ⁻¹ · ml ⁻¹)	Activity (nmol h ⁻¹ · mg protein ⁻¹)
1 mM NaNO ₃ -10% CH ₄	3.2 ± 0.2	4.5 ± 0.3
1 mM NaNO ₃ -10% CH ₄ -5% O ₂	7.4 ± 0.5	10.4 ± 0.7
10% CH ₄ -5% O ₂	8.4 ± 1.4	11.7 ± 1.9

^aCalculated per ml of reactor biomass and mg of protein.

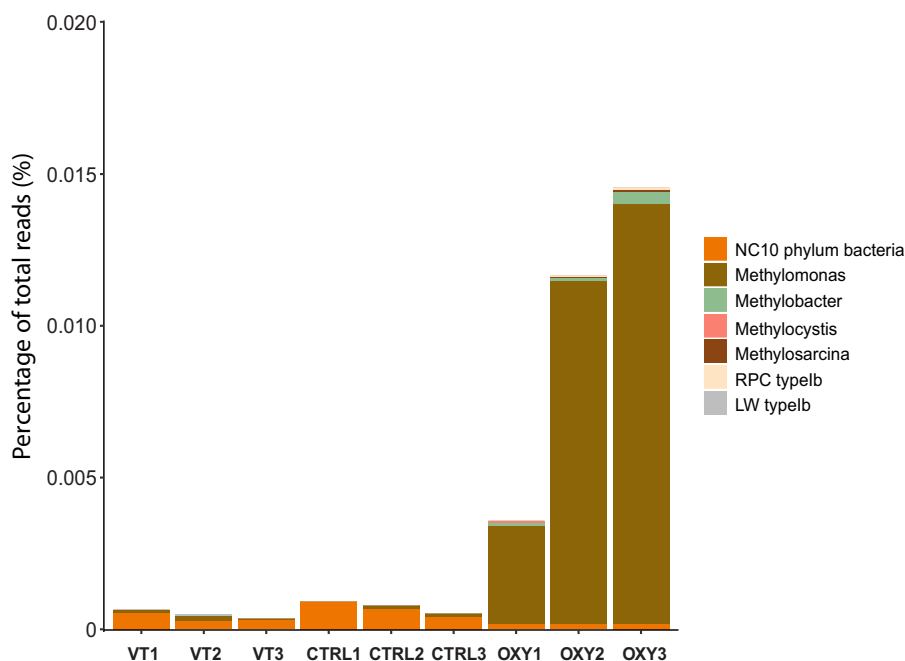


FIG 4 Taxonomic *pmoA* gene read distribution obtained from metatranscriptome (VT) sequencing of the bioreactor and metatranscriptome sequencing of batch incubations supplied with 1 mM nitrate and 10% methane in the headspace (CTRL) and 1 mM nitrate and 10% methane and 5% oxygen in the headspace (OXY). The three replicates are shown separately.

incubations with oxygen, *pmoA* reads assigned as *Methylomonas* sp. increased three orders of magnitude, whereas no increase in read numbers of *pmoA* of NC10 phylum bacteria was observed.

Changes in transcript levels of "*Ca. Methanoperedens nitroreducens Verserenetto*" upon exposure to oxygen. We compared the changes in gene expression levels in anoxic control incubations (with nitrate and methane) versus those in incubations with oxygen exposure (nitrate, methane, and oxygen). Out of 3,873 transcripts, 1,052 were upregulated, 508 were downregulated (2-fold threshold for upregulation or downregulation), and 583 showed no change in expression levels in oxygen-amended incubations. The results of the analysis are shown for central energy metabolism of "*Ca. Methanoperedens nitroreducens Verserenetto*," targeting the key functional genes encoding methane oxidation and nitrate reduction pathways (Table 2), as well as for potential genes encoding oxidative stress response pathways (Table 3).

For the methane oxidation pathway, 13 transcripts out of 23 transcripts were downregulated. Most downregulated genes encoded the MCR enzyme complex, with *mcrG* and *mcrA* showing -8.75 - and -8.63 -fold changes upon oxygen exposure, respectively. In the nitrate reduction pathway, the most downregulated gene was nitrite reductase *nrfA* (-18.64 -fold change), followed by nitrate reductase *narG* (-4.53 -fold change).

The genome of "*Ca. Methanoperedens nitroreducens Verserenetto*" contains several putative oxidative stress genes (Table 3). A total of 14 out of 34 of these genes showed upregulation after oxygen exposure. Upon oxygen exposure, there was clear upregulation of genes encoding glutaredoxin, thioredoxin family/like proteins, rubrerythrins, peroxiredoxins, peroxidase, alkyl hydroperoxidase, type A flavoproteins, Fe-S cluster assembly protein, and cysteine desulfurases. Furthermore, in the genome of "*Ca. Methanoperedens nitroreducens Verserenetto*," several putative genes possibly involved in archaeum formation were identified (Table S1) and *flaI*, *flaD*, *flaJ*, *flaB*, and *flaK* showed 2- to 16-fold upregulation upon oxygen exposure.

TABLE 2 Difference in gene expression of genes encoding proteins involved in the methane oxidation and denitrification pathway in oxygen-amended incubations versus anoxic control incubations after 24 h

Gene name ^a	Gene abbreviation	Annotation	Fold change	P value
Methyl-coenzyme M reductase	<i>mcrG</i>	MNVS_02828	-8.75	2.52E-19
	<i>mcrA</i>	MNVS_02829	-8.63	1.95E-19
	<i>mcrB</i>	MNVS_02826	-7.73	7.45E-17
	<i>mcrC</i>	MNVS_02794	-3.27	9.69E-04
Tetrahydromethanopterin S-methyltransferase	<i>mtrF</i>	MNVS_02473	-4.59	3.53E-01
	<i>mtrC</i>	MNVS_02476	-2.89	9.23E-02
	<i>mtrA1</i>	MNVS_02474	-2.85	2.12E-02
	<i>mtrG</i>	MNVS_02472	-2.51	3.47E-01
	<i>mtrD1</i>	MNVS_02477	-2.39	1.99E-01
	<i>mtrB</i>	MNVS_02475	-2.14	4.22E-01
	<i>mtrH</i>	MNVS_02471	-1.97	8.09E-02
	<i>mtrE</i>	MNVS_02478	-1.33	6.34E-01
	<i>mtrD2</i>	MNVS_02633	2.97	3.70E-01
F ₄₂₀ -dependent methylene-H4MPT reductase	<i>mer</i>	MNVS_02157	-1.58	2.42E-01
F ₄₂₀ -dependent methylene-H4MPT dehydrogenase	<i>mtd</i>	MNVS_00113	-4.53	6.61E-03
Methenyl-H4MPT cyclohydrolase	<i>mch_1</i>	MNVS_00232	-4.63	2.25E-01
	<i>mch_2</i>	MNVS_01068	1.40	6.94E-01
Tetrahydromethanopterin formyltransferase	<i>ptr</i>	MNVS_01136	1.06	9.22E-01
Molybdenum-dependent formyl-MFR dehydrogenase	<i>fmdC_1</i>	MNVS_01939	-11.88	5.59E-04
	<i>fmdC_2</i>	MNVS_02819	1.48	7.71E-01
Tungsten dependent formyl-MFR dehydrogenase	<i>fwdC_2</i>	MNVS_01111	-1.80	7.82E-01
	<i>fwdC_3</i>	MNVS_02822	-1.53	4.47E-01
	<i>fwdC_1</i>	MNVS_00424	1.42	7.35E-01
Nitrate reductase	<i>narG_1</i>	MNVS_03649	-4.53	6.87E-04
	<i>narG_2</i>	MNVS_03754	-3.78	2.44E-06
	<i>narH_2</i>	MNVS_03753	-3.78	1.31E-03
	<i>narH_1</i>	MNVS_03650	-3.34	4.43E-02
	<i>narC_1</i>	MNVS_01288	-2.68	3.00E-01
	<i>narB_1</i>	MNVS_01290	-1.13	8.88E-01
	<i>narC_2</i>	MNVS_01289	1.15	8.50E-01
Formate-dependent nitrite reductase	<i>nrfA</i>	MNVS_03633	-18.64	3.65E-05

^aH4MPT, tetrahydromethanopterin; MFR, methanofuran.

DISCUSSION

In marine environments, up to 90% of the methane produced in deeper layers is consumed by anaerobic, sulfate-dependent methane oxidation before it is released to the atmosphere (53). In freshwater ecosystems, sulfate concentrations are typically lower, and nitrogen oxides may serve as alternative electron acceptors for microorganisms performing nitrate- or nitrite-dependent anaerobic oxidation of methane. However, due to the few available studies, the contribution of N-AOM microorganisms to global methane mitigation in comparison to that of aerobic methanotrophs is not yet resolved. In natural or man-made ecosystems, oxic and anoxic interphases such as semi-oxic rhizosphere and soil in paddy fields, often cooccur and provide a niche for both aerobic and anaerobic methanotrophs. Thus, for estimations of the contribution of anaerobic methanotrophs to methane mitigation in natural environments, as well as for possible application in wastewater treatment plants, the tolerance and response of N-AOM microorganisms to oxygen is of great relevance.

Physiological studies of N-AOM microorganisms are challenging, as both organisms grow very slowly and have a doubling time of days to weeks, and no pure cultures exist. In this study, we investigated the response of a culture highly enriched with a new N-AOM archaeon strain, "*Ca. Methanoperedens nitroreducens Verserenetto*," upon exposure to oxygen. The current enrichment is one of the highest enrichments re-

TABLE 3 Differences in gene expression for genes encoding proteins involved in oxidative stress response pathways in oxygen-amended incubations versus anoxic control incubations after 24 h

Gene name	Gene/protein abbreviation ^a	Annotation	Fold change	P value
Superoxide dismutase FE	Sod	MNV5_00641	-2.75	3.70E-03
Superoxide dismutase FE	Sod	MNV5_03558	1.13	9.45E-01
Superoxide dismutase FE	Sod	MNV5_03536	-3.30	6.73E-01
Glutaredoxin	Grx	MNV5_02652	2.32	7.24E-01
Thioredoxin	Trx	MNV5_01832	1.05	9.59E-01
Thioredoxin	Trx	MNV5_01004	1.03	9.69E-01
Thioredoxin family protein	-	MNV5_01348	8.22	1.29E-01
Thioredoxin family protein	-	MNV5_00830	-3.97	1.64E-01
Thioredoxin-like protein	-	MNV5_03051	1.51	7.60E-01
Ferredoxin-thioredoxin reductase	TrxR	MNV5_03540	1.16	9.16E-01
Rubryerythrin	Rbr	MNV5_03304	1.46	5.61E-01
Rubryerythrin	Rbr	MNV5_03551	1.80	3.98E-01
Reverse rubryerythrin (rubryerythrin family protein)	Rbr	MNV5_00963	9.45	3.48E-03
Rubredoxin	Rub	MNV5_01468	-4.59	3.50E-01
Rubredoxin	Rub	MNV5_02621	-3.53	5.66E-01
Rubredoxin	Rub	MNV5_02800	-2.13	6.54E-01
Peroxiredoxin	Prx	MNV5_02094	1.99	5.89E-01
Peroxiredoxin	Prx	MNV5_03554	1.32	1.61E-01
Peroxiredoxin	Prx	MNV5_01833	-1.85	7.61E-01
OsmC family peroxiredoxin	Prx	MNV5_01176	1.61	7.06E-01
Peroxidase	Px	MNV5_02565	5.51	6.25E-05
Alkyl hydroperoxidase	AhpD	MNV5_00489	1.97	6.90E-01
Type A flavoprotein FprA	FprA	MNV5_01083	22.16	1.11E-02
Type A flavoprotein FprA	FprA	MNV5_03305	1.06	7.72E-01
Type A flavoprotein FprA	FprA	MNV5_02778	-1.48	5.25E-01
Type A flavoprotein FprA	FprA	MNV5_01610	-1.02	5.24E-01
Type A flavoprotein FprA	FprA	MNV5_01745	3.80	2.32E-02
Type A flavoprotein FprA	FprA	MNV5_00485	-1.32	7.40E-01
Type A flavoprotein FprA	FprA	MNV5_00503	-1.65	3.10E-01
Iron-sulfur cluster assembly protein	NifU	MNV5_01057	1.76	5.26E-02
Iron-sulfur cluster assembly protein	NifU	MNV5_01084	1.17	7.24E-02
Cysteine desulfurase	NifS	MNV5_01058	2.00	3.57E-01
Cysteine desulfurase	NifS	MNV5_01085	1.64	3.15E-01
Cysteine desulfurase	sufS	MNV5_00788	-1.07	9.23E-01

^a-, no gene/protein name could be assigned.

ported for this microorganism, with an 83% dominance of this strain in the enrichment culture. Despite the high enrichment of "Ca. Methanoperedens nitroreducens," it is important to take into consideration the other minor yet diverse members in the microbial side community, consisting of microorganisms that possibly convert methane, nitrogen oxides, and organic carbon. Despite the long anoxic incubation time (>2 years), this community still contained a population of aerobic methane-oxidizing *Methylomonas* bacteria (approximately 0.1% of the 16S rRNA reads of the bioreactor and control incubation metatranscriptome), which are presumably inactive but which become active (approximately 7% of the 16S rRNA reads of oxygen-amended incubation metatranscriptome) and oxidize methane as soon as (traces of) oxygen become available (54). It may be that these aerobic methanotrophs are also active without the presence of oxygen or at very low oxygen concentrations, as has been demonstrated for several oxygen-limited environments, where aerobic methanotrophs are abundant and active (55–57). Furthermore, some MOB couple methane oxidation to nitrate reduction under hypoxic conditions (58) and may carry out fermentation during oxygen starvation (59). Similar observations were made for the persistent presence of aerobic ammonium oxidizers in anammox enrichment cultures (60, 61).

Transcriptome analysis revealed that oxygen exposure substantially altered the composition of active members of the community in the enrichment culture. The 16S rRNA gene transcription was downregulated by 4% for "Ca. Methanoperedens nitroreducens Verserenetto" in incubations with added oxygen versus that in anoxic control incubations. "Ca. Methanoperedens nitroreducens" archaea are closely related to methanogens and have a similar metabolic pathway that operates in a reverse direction

(Table 2 summarizes the genes involved in this pathway). Since methanogens are considered to be very sensitive to oxygen and become inhibited upon exposure to it (62), "*Ca. Methanoperedens nitroreducens*" was hypothesized to exhibit a similar reaction. The exposure to oxygen resulted in a large-scale downregulation of key metabolic genes involved in the reverse methanogenesis and nitrate reduction pathway. The central dissimilatory nitrogen metabolism in "*Ca. Methanoperedens nitroreducens*" is represented by reduction of nitrate to nitrite, although in addition, there is genomic and experimental evidence for a potential dissimilatory nitrite reduction to ammonia (DNRA; *nrfAH*) (63, 64). The potential for DNRA and the conditions under which it occurs have not been investigated in detail and are challenging due to presence of nitrite-scavenging bacteria in the microbial communities (4–6). The genes encoding key enzymes involved in nitrate reduction (*narG*) and DNRA (*nrfA*) were strongly downregulated in "*Ca. Methanoperedens nitroreducens* Verserenetto" exposed to oxygen. However, oxygenic heterotrophic denitrification may still occur under microaerophilic or even oxic conditions (65), thus making a closed nitrogen balance challenging.

Regarding the capability to endure oxygen exposure, the genome of "*Ca. Methanoperedens nitroreducens* Verserenetto" contained several putative genes involved in counteracting oxidative stress. We identified three gene copies of superoxide dismutase, one glutaredoxin, two thioredoxins, three thioredoxin family/like proteins, ferredoxin-thioredoxin reductase, three rubrerythrins, two peroxiredoxins, three rubredoxins, four peroxiredoxins, one peroxidase, one alkyl hydroperoxidase, seven type A flavoproteins, three cysteine desulfurases, and two Fe-S cluster assembly proteins. Stress response to oxygen has been extensively studied in methanogens (62), which are the closest relatives of "*Ca. Methanoperedens nitroreducens*." Despite their strictly anaerobic lifestyle and sensitivity to oxygen, there is an increasing amount of evidence that methanogens are present in semi-oxic soils and are able to resume methanogenic activity once anoxic conditions are restored (41). Furthermore, the detection of methanogens and, by functional inclusion, methanotrophic archaea in oxic environments has been reported with higher frequency than expected (66). It has been shown that the methanogen *Methanobrevibacter cuticularis* is even able to consume oxygen at low concentrations while producing methane (67). The view of methanogens from the genera *Methanosarcina* and *Methanocella* being strict anaerobes has shifted toward one of "universal members of aerated soils" (41). Some methanogens are able to counteract oxidative stress, which allows them to exist in semi-oxic and aerated environments (48, 49). Recently, analysis of the genomic potential of several methanogens revealed the presence of genes encoding enzymes involved in oxidative stress defense.

The transcriptome data of our oxygen-exposed batch incubations indicated that upregulation of thioredoxin-like proteins, thioredoxin reductase, thioredoxin, glutaredoxin, rubrerythrins, peroxiredoxins, peroxidase, alkyl hydroperoxidase, type A flavoproteins, Fe-S cluster assembly protein, and cysteine desulfurase occurred in "*Ca. Methanoperedens nitroreducens* Verserenetto" upon exposure to oxygen.

Thioredoxin (Trx) is a small, well-characterized protein which is nearly universal in methanogens and is involved in response to oxidative stress (68). *Methanosarcina acetivorans* harbors seven Trx homologues, whereas members of the *Methanococcus* and *Methanobacteria* genera harbor two (69). In methanogens that actively produce methane in oxygenated soils, such as "*Candidatus* Methanotrix paradoxum," two copies of rubrerythrin genes have been described and shown to be highly transcribed (44). In the case of "*Ca. Methanoperedens nitroreducens*" archaea and their response to oxygen exposure, the upregulation of thioredoxin- and glutaredoxin-encoding genes suggests a self-repairing strategy against oxidative stress, which is characteristic for the class II methanogens to which "*Ca. Methanoperedens nitroreducens*" is most closely affiliated. The expression of thioredoxins is known to be correlated to the repair mechanisms that take place after the oxidation of membrane phospholipids and oxidation of pyrimidines; these self-repair mechanisms consists of the reduction of phospholipid hydroperoxides, reduction of disulfide bonds, and the assembly of Fe-S

clusters (45). Fe-S cluster assembly systems are encoded by several strains of *Methanocellales*, which are known for their aerotolerant lifestyle (70). Furthermore, genes encoding cysteine desulfurases were present in the genome of "*Ca. Methanoperedens nitroreducens*," with one copy upregulated upon oxygen exposure. These are likely linked to scavenging of sulfur from amino acids containing cysteine and are needed for the assembly and repair of oxidized Fe-S clusters. Alkyl hydroperoxidases convert the endogenously produced hydrogen peroxide to water (71). Similar to hydroperoxidases, rubrerythrins serve in deactivating the ROS, by neutralizing them with water as end product. The upregulation of genes encoding rubrerythrins and hydroperoxidases suggests that "*Ca. Methanoperedens nitroreducens*" generally employs the ROS deactivation strategy without internal oxygen production (72). The self-repair response and the deactivation of oxygenic mechanisms is enhanced by the upregulation of genes encoding type A flavoproteins, known to be involved in chain reactions for oxygen reduction. This mechanism has been studied for obligate anaerobic bacteria from the genus *Clostridium*, and it is generally involved in the maintenance of the redox balance by influencing the ratio of NADP⁺/NADPH in cellular processes (73, 74).

Besides the upregulation of genes directly involved in counteracting oxygen damage, several putative genes involved in archaellum formation were upregulated (2- to 16-fold) in oxygen-exposed "*Ca. Methanoperedens nitroreducens* Verserenetto." The transcription of putative genes encoding FlaD (signaling component), FlaB2_2 (encoding archeallin), FlaK (pre-flagellin peptidase, cleaves the pre-flagellin leader peptide) and FlaI (homologue of the PilB-like polymerizing ATPase, a key component of the type IV pili assembly system) (75–77), indicates that these archaea might be able to form archaella in response to adverse conditions. Biosynthesis of archaella will need to be confirmed by proteomic and electron microscopy studies.

Besides the anaerobe "*Ca. Methanoperedens nitroreducens*," the microbial community contained NC10 phylum bacteria (1.9% of the 16S rRNA gene reads in the metagenome). From the genus *Methylomirabilis*, NC10 phylum bacteria use nitrite as an electron acceptor, which they may receive either from "*Ca. Methanoperedens nitroreducens* Verserenetto" or from heterotrophic nitrate reducers. The methanotrophic NC10 phylum bacteria with their putative intraaerobic pathway seemed not to respond to the addition of oxygen in the investigated time frame, since in the metatranscriptomes from both treatments, the total 16S rRNA gene reads assigned to these bacteria remained at about 6%. However, the comparison of NC10 *pmoA* transcript levels in control and oxic batch incubations revealed a decrease from $7 \times 10^{-4}\%$ to $2 \times 10^{-4}\%$, indicating a reduced methanotrophic activity of NC10 phylum bacteria. The effect of oxygen exposure on an enrichment culture dominated by NC10 phylum methanotrophs was previously investigated (30). In that study, biomass was exposed to 2% and 8% oxygen, which resulted in a decrease in methanotrophic activity, as well as downregulation of key genes encoding enzymes in methane oxidation and nitrite reduction pathways after 24 h of exposure (30). Thus, the reduced expression of NC10 bacterial *pmoA* observed in this study correlates with previous findings. The gene involved in putative nitric oxide dismutation to nitrogen gas, *norZ2*, was the only copy that showed upregulation; however, the role of these enzymes remains to be experimentally validated. The effect of lower oxygen concentrations, namely, 0.7%, 1.1%, and 2% oxygen, have been investigated in enrichment cultures of NC10 phylum bacteria, and only 2% oxygen resulted in a decrease in methanotrophic activity and nitrite reduction activity. Unexpectedly, 1.1% oxygen increased methanotrophic activity and even nitrite reduction. This increase in activity could be related to NC10 phylum bacteria activity; however, *Gammaproteobacteria* were present in an increased amount in the enrichment culture, based on fluorescence *in-situ* hybridization. However, these proteobacteria are known to include heterotrophic denitrifiers (31).

Both, 16S rRNA and *pmoA* gene analyses also revealed a significant stimulation of MOB in oxic conditions versus that in the control incubations. The most dominant MOB was represented by a *Methylomonas* sp. type I methanotroph regularly found in both marine and freshwater environments, including paddy fields (78–82). Although the

abundance of these bacteria in the enrichment culture was very low (0.1% of 16S rRNA reads) in the reactor and batch incubation metatranscriptomes, it increased to 7% of total 16S rRNA reads in oxic batch incubation metatranscriptomes. The presence of *Methylomonas* species in anaerobic methane-oxidizing enrichments has been reported before (54), as has an increase in methanotrophic activity of anaerobic cultures upon oxygen exposure (31). Aerobic methanotrophs are known to rapidly become active under suitable conditions. Our activity assays supported the transcriptome data, showing that total methanotrophic activity in oxic incubations exhibited a lag phase of 6 h, after which the methane conversion rate started to increase and surpassed the rate of anoxic control incubations after 18 h. The persistence of aerobic methanotrophs in long-term anoxic conditions and their much higher affinity for methane creates challenges in assessing the contribution of anaerobic methane oxidizers, even in anoxic bioreactors. However small the relative abundance of a microorganism is in the microbial community, it may account for a large extent of activity. Furthermore, the competition in anoxic environments between MOB and N-AOM microorganisms for methane when traces of oxygen become available might be driven by their abilities to consume methane at different rates. ANME archaea carrying out sulfate-dependent AOM exhibit an affinity constant for methane in the millimolar range (83), whereas some MOB have extremely low affinity constants, in the nanomolar range (84). Another factor that drives the shift from aerobic to anaerobic methanotrophy may be the oscillating anoxic conditions in environments such as paddy fields, where the agricultural runoff provides nitrogen-based electron acceptors, which become more abundant than oxygen.

In conclusion, a new strain (84), "*Ca. Methanoperedens nitroreducens Verserenetto*" was enriched and sequenced. This study showed a strong response of the newly enriched anaerobic methanotrophic archaea to exposure of oxygen, based on measured activities and gene expression analysis, and the oxidative stress response relies on the expression of highly reducing enzymes, which are involved in self-repair upon oxygen stress. Thus, transient exposure to oxygen for longer times, as might occur at suboxic interfaces in soils and wastewater treatment plants, would lead to inactivation of anaerobic methanotroph activity and a rapid growth of aerobic methanotrophs. It remains to be investigated how "*Ca. Methanoperedens nitroreducens*" would respond to shorter exposure time at lower micromolar concentrations of oxygen, mimicking processes that occur at root-soil interfaces in ecosystems such as paddy fields.

MATERIALS AND METHODS

Enrichment culture. A 5-liter bioreactor (Applikon, Schiedam, the Netherlands) was started with biomass from an enrichment culture of "*Ca. Methanoperedens nitroreducens*" described previously by Vaksmaa et al. (6) and operated at room temperature as a sequencing batch reactor for 8 months. The sequence consisted of 12-h cycles, with 10 h of constant medium supply, 1 h of settling of the biomass, and 1 h of removal of excess liquid. The medium composition was $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (0.1 g/liter) and KH_2PO_4 (0.05 g/liter), and it was autoclaved before the addition of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.07 g/liter); 5 ml of a trace element stock solution (in 10 liters of medium) composed of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.438 g/liter), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.6g/liter), CuSO_4 (4 g/liter), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.95 g/liter), H_3BO_3 (0.07 g/liter), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1 g/liter), $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ (0.1 g/liter), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.5 g/liter), SeO_2 (0.134 g/liter), and CeCl_2 (0.11 g/liter); 3 ml of an iron stock solution composed of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (32.4 g/liter) and nitrilotriacetic acid (NTA; 96 g/liter), and 1 ml of vitamin solution (DSMZ 141). NaNO_3 concentration in the medium was increased from 2.5 mM to 5 mM after six months of operation due to the increased consumption rate. The medium was continuously sparged with argon- CO_2 (95% vol/vol). The bioreactor was operated at pH 7.25, stirred at 150 rpm, and sparged with $\text{CH}_4\text{-CO}_2$ (95% vol/vol).

DNA extraction. DNA extraction was performed on a 10-ml sample of reactor biomass at the start of batch incubations, using the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's protocol. A first step with rigorous bead beating for 3 min at 30 beats/s was needed to disrupt the archaeal cells. DNA quantity and quality were assessed by UV-visible (UV-VIS) spectroscopy (NanoDrop, ND-1000; Isogen Life Science, the Netherlands) and Qubit double-stranded DNA (dsDNA) high-sensitivity assay kit (Agilent Technologies, Santa Clara, CA).

Metagenome sequencing and data analysis. For library preparation, 1 ng DNA in total was used with the Nextera XT kit (Illumina, San Diego, CA). Enzymatic fragmentation was performed, followed by incorporation of the indexed adapters and amplification of the library. Purification of the amplified library was performed using Agencourt AMPure XP beads (Beckman Coulter Nederland B.V.), and quality and size distribution were checked using the Agilent 2100 Bioanalyzer and the high-sensitivity DNA kit

(Agilent Technologies, Santa Clara, CA). Fluorometric quantitation of the library was performed by Qubit, using the Qubit dsDNA high-sensitivity (HS) assay kit (Thermo Fisher Scientific Inc., Waltham, MA). For normalization of the library, the concentration measured by a Qubit fluorometer and the average fragment size obtained with the Agilent 2100 Bioanalyzer were used to calculate the correct dilution factor. After denaturing and dilution to 4 nM using the Denature and Dilute Libraries Guide (Illumina, San Diego, CA), the library was sequenced using an Illumina MiSeq machine (San Diego, CA). Paired-end sequencing was performed with 2×300 -bp sequence chemistry, using the MiSeq reagent kit v3 (San Diego, CA) according to the manufacturer's protocol.

Obtained reads were quality trimmed using CLC Genomics Workbench 11 (Qiagen Aarhus A/S) with standard settings (minimal fragment length of 50 bp, trimming of the first 20 nucleotides at the 5' site, and a quality score limit of 0.01). Paired reads were merged using the standard settings, and all merged reads, "orphans," and single paired reads were included into a *de novo* assembly (default settings; word size, 30; bubble size, 5,000; length fraction, 0.5; similarity fraction, 0.95; minimum contig length, 1,000 bp). To extract the contigs of "*Candidatus* Methanoperedens nitroreducens Verserenetto," the contigs were binned based on GC content and read coverage in Rstudio (85). The contigs of "*Ca. Methanoperedens nitroreducens Verserenetto*" were extracted, and the completeness of the draft genome and contamination were assessed by CheckM (16). Prokka was used for annotation of the genome of "*Ca. Methanoperedens nitroreducens Verserenetto*" (19). Metagenomic data have been deposited in the European Nucleotide Archive (ENA) under ENA accession number [ERS2523352](https://ena.ebi.ac.uk/ena/record/ERS2523352) (NCBI BioSample number [SAMEA4703186](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4703186)). The draft genome sequence of "*Ca. Methanoperedens nitroreducens Verserenetto*" has been deposited in the European Nucleotide Archive under ENA accession number [ERS2527105](https://ena.ebi.ac.uk/ena/record/ERS2527105) (NCBI BioSample number [SAMEA4706944](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4706944)).

The 16S rRNA genes were retrieved from the metagenome by mapping all reads to the SILVA 128 SSURef Nr99 database (insertion cost, 3; deletion cost, 3; length fraction, 0.5; and similarity fraction, 0.95). All mapped sequence reads were further processed by the next-generation sequencing (NGS) analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (86). Each read was aligned using the SILVA Incremental Aligner (SINA; SINA v1.2.10 for ARB SVN [21008]) (87) against the SILVA small subunit (SSU) rRNA SEED and quality controlled (86). Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities or 2% of homopolymers were excluded from further processing. Putative contaminations and artifacts, namely, reads with a low alignment quality (alignment identity of 50 and alignment score of 40 reported by SINA), were identified and excluded from downstream analysis. After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered into operational taxonomic units (OTUs) on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering were done using *cd-hit-test* (v 3.1.2; <http://www.bioinformatics.org/cd-hit>) (88) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the nonredundant version of the SILVA SSU Ref data set (release 132; <http://www.arb-silva.de>) using BLASTN (version 2.2.30+; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with standard settings (89). The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. Reads without any BLAST hits or reads with weak BLAST hits, where the function " $(\% \text{ sequence identity} + \% \text{ alignment coverage})/2$ " did not exceed the value of 93, remain unclassified. These reads were assigned to the meta group "No Relative" in the SILVAngs fingerprint and Krona charts (90). This method was first used in the publications of Klindworth et al. (91) and Ionescu et al. (92).

Fluorescence *in situ* hybridization. Prior to the incubation experiments, 1.5 ml of reactor biomass was harvested for fluorescent *in situ* hybridization (FISH). The biomass, which consisted of dense granular aggregates, was first disrupted by mechanical force through a series of needles with decreasing diameter, as follows: 1.2, 0.8, 0.6, and 0.4 mm. The disrupted biomass was then pelleted by centrifugation at $4,000 \times g$ for 5 min, and the supernatant was discarded. The pellet was washed twice with 1 ml phosphate-buffered saline (PBS; 130 mM NaCl and 10 mM phosphate buffer [pH 7.4]). After the washing steps, the pellet was resuspended in 0.3 ml of PBS and fixed with 900 μ l paraformaldehyde (4%) overnight at 4°C. FISH was performed as previously described (2), using 35% formamide stringency. The specific probes used were DAMOBACT-0193 (5'-CGC TCG CCC CCT TTG GTC-3') for *Methyloirabilis*-like bacteria; DAMOARCH-0641 (5'-GGT CCC AAG CCT ACC AGT-3') for "*Ca. Methanoperedens nitroreducens*" archaea; EUB 338 (S-d-Bact-0338-a-A-18) (93), EUB 338 II (S-d-Bact_0338-b-A-18) (94), and EUB 338 III (S-d-Bact-0338-c-A-18) (94) for most bacteria; and S-d-Arch-0915-a-A-20 (95) for most archaea. Vectashield as mounting medium with 4',6-diamidino-2-phenylindole (DAPI; 5 mg/ml) was used as counter stain. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with a charge-coupled device (CCD) camera, together with the Axiovision software package (Zeiss, Germany).

Activity measurements. Activity assays were performed in 60-ml serum bottles containing 45 ml of reactor biomass. Prior to sampling, the biomass in the reactor was stirred at 500 rpm for 5 min to ensure homogeneity. After transfer, $^{15}\text{N-NaNO}_3$ (final concentration in the bottles, 1 mM) was added; controls received no addition. The bottles were sealed with red butyl rubber stoppers and crimp capped. The bottles were made anoxic by five 3-min cycles of vacuum and purging with argon- CO_2 (95% to 5%). One bar of overpressure was introduced to the bottles, and 10% $^{13}\text{C-CH}_4$ was added to all bottles, except for the controls without methane. Oxygen (5%) was added to the headspace of incubations of oxidative stress. Gas samples of 50 μ l were taken at various time points over a 24-h period. The production of $^{13}\text{C-CO}_2$ and consumption of O_2 were monitored by gas chromatography-mass spectrometry (GC-MS) (Agilent 5975 inert MSD; Agilent, USA). Calibration was performed with standard gas consisting of $\text{QS}/1.06\%/0.82\%/1.32\%/459 \text{ ppm He}/\text{CO}_2/\text{N}_2/\text{O}_2/\text{N}_2\text{O}$ (Air Liquide BV, the Netherlands).

Protein determination. Protein determination was carried out on 10-ml reactor biomass samples, in duplicate. Samples were taken after settling in the sequencing batch reactor (SBR) cycle and potted using a glass homogenizer. A volume of the homogenized sample was mixed 1:1 with a 3 M NaOH solution and heated for 30 min at 90°C. After cooling to room temperature, CuSO₄ 4% was added in a 2:1 ratio, vigorously mixed, and centrifuged at 4,000 × *g* for 5 min at room temperature (RT). The supernatant was transferred and measured at 540 nm (Spectronic 200; Thermo Scientific, USA). Bovine serum albumin was used for the calibration curve.

RNA extraction. RNA was extracted from each of the incubation assays (control with 1 mM nitrate and 10% methane and oxygen exposed with 1 mM nitrate, 10% methane, and 5% oxygen) after 24 h and simultaneously from the reactor biomass in triplicate, using the Ribopure RNA extraction kit according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). An additional bead beating step of 7 min of 30 beats/s was added to disrupt the cells. RNA was eluted from the spin column with 25 μl of diethyl pyrocarbonate (DEPC)-treated MilliQ water. Quality and quantity were determined with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a Qubit fluorometer using the Qubit RNA HS assay kit (Thermo Fisher Scientific, Inc., Waltham, MA), respectively.

Metatranscriptome sequencing and data analysis. For the transcriptome libraries, the TruSeq stranded mRNA sample preparation protocol was used according to the manufacturer's instructions (Illumina, San Diego, CA). Minor adaptations to the protocol were introduced, since total RNA was used for library preparation. The libraries obtained were quantitatively and qualitatively checked and processed as described above. After dilution to 4 nM, pooling the libraries, and denaturing using the Denature and Dilute Libraries Guide (Illumina, San Diego, CA), the libraries were sequenced on the Illumina MiSeq machine (San Diego, CA). Pooled libraries were loaded in the cartridge, and sequencing was performed with 150-bp sequence chemistry using the MiSeq reagent kit v3 (San Diego, CA) according to the manufacturer's protocol in one direction.

To analyze the metatranscriptome, trimmed reads were mapped to the annotated draft genome of "*Ca. Methanoperedens nitroreducens Verserenetto*" (see above) using CLC Genomics Workbench 11 (transcriptome sequencing [RNA-seq]) and further analyzed using the R package "Deseq2" (96).

To analyze the 16S rRNA genes from the active community, reads from the metatranscriptomes were trimmed (default settings, quality limit of 0.01 and minimal length of 50 bp) using the CLC Genomics Workbench 11 (Qiagen Aarhus A/S) and mapped against the SILVA 128 SSURef Nr99 database (insertion cost, 3; deletion cost, 3; length fraction, 0.5; similarity fraction, 0.98). Analysis was carried out as described above for the metagenomic sequencing, with the exception that subsampling per treatment was carried out, and 15,000 sequences were subsampled and analyzed as described above. Metatranscriptome data have been deposited in the European Nucleotide Archive (ENA) under ENA accession number [ERS2527068](https://ena.ebi.ac.uk/ena/record/ERS2527068) (BioSample number [SAMEA4706907](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4706907)).

pmoA gene read analysis. Quality-trimmed paired and merged metagenome reads and quality-trimmed metatranscriptome reads from all treatments were subjected to BLASTX analysis against a previously described, manually curated protein database for particulate methane monooxygenases and both archaeal and bacterial ammonia monooxygenases (cutoff E value, 10⁻⁶) (97). Positive reads were extracted and subjected to a BLASTX analysis against a nonredundant protein database. The outputs from both BLASTX analyses were plotted against each other based on calculated bit scores to obtain a bit score ratio. The bit score ratio was then used to filter out false-positive hits using a procedure described previously (20). Positive hits were then classified in MEGAN (version 5.11.3) based on the procedure and database described previously (98, 99).

Data availability. Metagenomic data have been deposited in the European Nucleotide Archive (ENA) under the ENA accession numbers [ERS2523352](https://ena.ebi.ac.uk/ena/record/ERS2523352) (NCBI BioSample number [SAMEA4703186](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4703186)), [ERS2527105](https://ena.ebi.ac.uk/ena/record/ERS2527105) ([SAMEA4706944](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4706944)), and [ERS2527068](https://ena.ebi.ac.uk/ena/record/ERS2527068) ([SAMEA4706907](https://www.ncbi.nlm.nih.gov/biosample/SAMEA4706907)).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01832-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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