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Metalloenzymes play major roles to achieve high-rate nitrogen removal in N-damo communities: Lessons from metaproteomics

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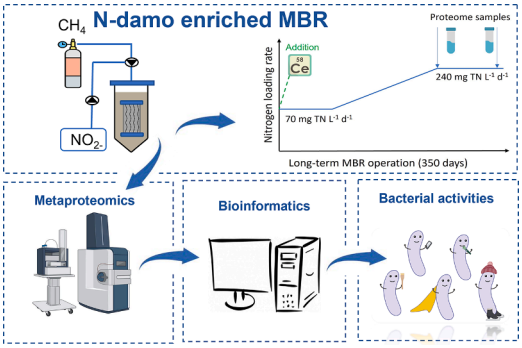
HIGHLIGHTS

- High NLR were achieved on a N-damo enriched MBR.
- Protein relative abundance showed that "*Ca.* M. lanthanidiphila" was predominant.
- The complete enzymatic machinery of N-damo bacteria for NO_2 and CH_4 was identified.
- Metaproteomics discovered the main activities of the accompanying taxa.
- Fe, Cu and Ce are crucial metals for optimal N-damo process performance.

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrite-driven anaerobic methane oxidation (N-damo) is a promising biological process to achieve carbon–neutral wastewater treatment solutions, aligned with the sustainable development goals. Here, the enzymatic activities in a membrane bioreactor highly enriched in N-damo bacteria operated at high nitrogen removal rates were investigated. Metaproteomic analyses, with a special focus on metalloenzymes, revealed the complete enzymatic route of N-damo including their unique nitric oxide dismutases. The relative protein abundance evidenced that "*Ca.* Methylomirabilis lanthanidiphila" was the predominant N-damo species, attributed to the induction of its lanthanide-binding methanol dehydrogenase in the presence of cerium. Metaproteomics also disclosed the activity of the accompanying taxa in denitrification, methylotrophy and methanotrophy. The most abundant functional metalloenzymes from this community require copper, iron, and cerium as cofactors which was correlated with the metal consumptions in the bioreactor. This study highlights the usefulness of metaproteomics for evaluating the enzymatic activities in engineering systems to optimize microbial management.

1. Introduction

Methane (CH_4) and nitrous oxide (N_2O) are two powerful greenhouse gases (GHGs) produced as microbial metabolism byproducts (Glass and Orphan, 2012; Stein, 2020). During biological wastewater treatment, CH_4 is the final product of anaerobic digestion, whereas N_2O

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is an intermediate product of heterotrophic denitrification of nitrite/nitrate to nitrogen gas (N_2) (Kampschreur et al., 2009). Mitigation of GHGs is critical for new wastewater treatment technologies adhering to sustainable goals (Ren et al., 2020).

Nitrite-driven anaerobic methane oxidation (N-damo) is a singular microbial process capable of removing CH_4 during a simultaneous denitrification without producing N_2O as intermediate, according to the following equation: $3\ CH_4 + 8\ NO_2^- + 8H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O$ ($\Delta G^\circ = -928$ kJ/mol CH_4) (Raghoebarsing et al., 2006; Ettwig et al., 2008). The only N-damo bacterial genus described to date is "*Candidatus* Methylomirabilis", and belongs to the NC10 phylum (Ettwig et al., 2008). Four species have been identified: "*Ca.* M. oxyfera" (Ettwig et al., 2010), "*Ca.* M. sinica" (He et al., 2016),"*Ca.* M. limnetica" (Graf et al., 2018) and"*Ca.* M. lanthanidiphila" (Versantvoort et al., 2018). Additionally, one genus of N-damo archaea was also described (i.e.: "*Ca.* Methanoperedens", ANME-2d clade) and shown to couple reverse methanogenesis to the use of nitrate as terminal electron acceptor (Haroon et al., 2013).

N-damo microorganisms are very attractive for sustainable wastewater treatment, and their biotechnological applications have demonstrated its efficiency to remove nitrogen in the form of nitrate (Cai et al., 2015) or nitrite up to competitive removal rates (e.g.: 285.7 mg N-$NO^{-2}L^{-1}d^{-1}$) (Arias et al., 2022). However, despite previous research efforts, full-scale implementation of the N-damo remains a challenge due to their slow growth (Ettwig et al., 2010), low biomass activities (Chang et al., 2021), strict anaerobic requirements and need for sufficient CH_4 availability (Chen et al., 2023).

A model for the methane activation and nitrite reduction metabolic pathways in "*Ca.* M. lanthanidiphila" was fully described in (Versantvoort et al., 2019, 2018). Their key reactions are catalyzed by specific enzymes that rely on the metal ion availability (Reimann et al., 2015) as was shown before in other microorganisms relying on respiratory chains that couple electron flow and proton translocation across their membranes (Budhraja et al., 2021; Reimann et al., 2015). Accordingly, a successful operational strategy to increase the N-damo activity was adjusting the mineral composition on the influent by increasing the bioavailability of trace metals which ultimately contributed to higher removal rates and a more stable operation (Arias et al., 2022; He et al., 2015; Reimann et al., 2015). Particularly, the dosage of Cu, Fe and Mo in adequate concentrations showed to significantly stimulate nitrite removal rates (NRR) (Arias et al., 2018; He et al., 2015; Jiang et al., 2018) by inducing the expression of functionally relevant genes, i.e.: those coding for nitrate reductases, nitric oxide reductases, methane monooxygenases and methanol dehydrogenases (Jiang et al., 2018; Reimann et al., 2015; Versantvoort et al., 2018). Additionally, the presence of lanthanides, such as cerium, have been shown to promote the activity of N-damo bacteria (Keltjens et al., 2014) particularly of "*Ca.* M. lanthanidiphila" (Guerrero-Cruz et al., 2019; Versantvoort et al., 2018; Quiton-Tapia et al., 2023).

N-damo species have not been isolated so far and previous works were done in enrichment cultures around 14% (J. Wang et al., 2019) to a maximum of 50% (Allegue et al., 2018) relative abundance of "*Ca.* Methylomirabilis". Other species might be outcompeting with N-damo for methane, nitrogen, and other components of the medium such as trace metals, therefore it is of great interest to find a tool to assess the activities of all the members of the community.

Mass spectrometry based metaproteomic emerged as a potent tool to discover the activities of specific microorganisms in mixed communities (Zhang et al., 2013). The technical advances achieved in the last decades allowed the identification of thousands of proteins from complex samples at once. Protein expressions are related to microbial activities more directly than gene detections (metagenomics) (Blakeley-Ruiz and Kleiner, 2022) or mRNA levels (metatranscriptomics). The latter comparison is especially relevant as mRNA levels do not always reveal the real bacterial activities (Wang et al., 2016). The relevance of metaproteomics to calculate the protein contribution of each member of the community was recently highlighted by Kleikamp et al., (2021) and Kleiner et al., (2017), and successfully applied in highly complex communities as aerobic activated sludge in Azizan et al., (2020) and Kennes-Veiga et al., (2022). The usefulness of metaproteomics to study enriched microbial communities in engineering systems is attracting biological wastewater researcher community (Kouba et al., 2022). In Wang et al., (2021), the authors showed how total annamox protein expressions remained stable with temperature while the key anammox proteins for nitrogen metabolism had a lower expression at 15 °C compared to 30 °C.

The aim of the present study was to evaluate the enzymatic activities of the different members of a highly enriched N-damo microbial community in a MBR during stable operation at a nitrite loading rate (NLR) $>$ 240 mg TN L^{-1}. d^{-1} with a special focus on metalloenzymes. For this purpose, shotgun metaproteomics were applied to the proteome of "*Ca.* Methylomirabilis" and its accompanying taxa. The corresponding results were related to metal concentrations in the reactor to assess their usefulness for process optimization stimulating the preferring N-damo pathways.

2. Material and methods

2.1. Reactor operation

The MBR, consisted in a 10 L glass reactor with a working volume of 6.65 L (Fig. 1). It contained a submerged hollow-fiber ultrafiltration membrane module (Puron) with a 0.5 m^2 surface and a pore size of 0.03 μm to retain the biomass. The membrane cycle operation consisted in 7 min of permeation and 30 s of relaxation, and the transmembrane pressure was continuously measured with a pressure sensor PN2069. Real time measurements for water level, gas volume flow, and redox potential were performed by in-situ sensors and collected by programable PLC Micro 820 (Allen-Bradley) connected to a computer. The inoculum was a complex microbial population that had been subject to previous enrichment strategies for>3 years and was enriched in 30–50% of *Ca.* Methylomirabilis (Allegue et al., 2018; Arias et al., 2022). The MBR system operated for about 350 days with a progressive increase in the nitrite loading rate (NLR).

The MBR was operated under anaerobic conditions. The liquid feed consisted of nitrogen as sodium nitrite in a nitrogen removal rate of 70 mg TNL^{-1} d^{-1} up to 240 mg TN L^{-1} d^{-1}. The feed also included a mix of

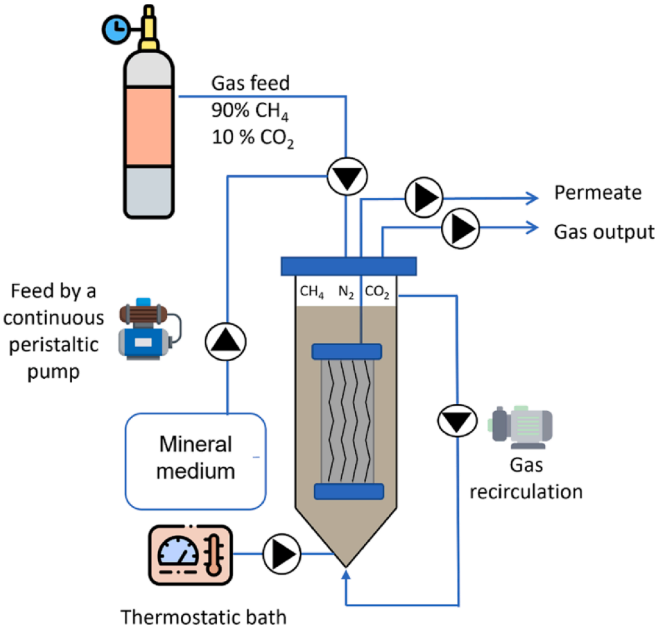

Fig. 1. Schematic representation of the N-damo enriched MBR reactor.

trace metals, EDTA as a chelating agent and other medium constituents as described in Arias et al., (2022), with the sole difference that cerium was included. In brief, relevant metals included in the medium were iron, copper, zinc, molybdenum, nickel, and cerium among others. The MBR was fed with the same medium composition a in a continuous flow mode during all the operation. The hydraulic retention time (HRT) was fixed in 1 day and temperature at 28 °C (using a thermostatic water bath). To maintain the HRT, peristaltic feeding pumps controlled in real time by a programmable PLC Micro 820 (Allen-Bradley) were operated to achieve a fixed permeate flow of 6.5 L d^{-1}. The system incorporated height sensors to signal any change in working volume, which in turn activated the feeding pumps. With the aim of enriching the N-damo population and maintaining stable conditions during our research the gas feed was supplied continuously from a gas mix bottle 90/10 CH_4/CO_2 v/v (Arias et al.2022; Quiton-Tapia et al., 2023). The gas flow was adjusted during the increase of NLRs to make the CO_2 counteract the alkaline production due to denitrification. The gas volume was adjusted to maintain an optimum pH (7.5 to 8) and the CO_2 supplied was equivalent to 32 mmol d^{-1} maximum, that is in the range of the observed overall nitrite removal in the system (~ 34 mmol d^{-1}).

2.2. Reactor performance monitoring

Nitrogen species (nitrite, nitrate and ammonia) were measured following spectrophotometric standard methods (APHA, 2000). Biomass concentration was quantified by means of total suspended solids (TSS) and volatile suspended solids (VSS). The redox potential and pH were measured daily to monitor the adequate functioning of the system. Biogas measurements were performed by gas chromatography using a 1 mL syringe to sample the outflow gas of the system. The gas volume fed to the system was measured using Mili GasCounter MGC-1 V3.3 PMMA (Ritter). Total organic carbon (TOC) was measured in the feed and permeate weekly by following standard methods (APHA, 2000) in a Shimazu TOC-L CSN.

Trace metal quantification was done by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). At the end of the reactor operation, when the reactor reached maximum performance, two samples taken in duplicates were extracted from the feed and permeate. Samples were extracted during stable reactor operation at days 300 and 335 for sample 1 and 2, respectively. Samples were then immediately filtered at 0.45 μm and frozen at –20 °C until analysis. The metals selected for analysis were those who were supplemented in the feeding medium: Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, W and Ce.

2.3. Proteomic analysis

2.3.1. Biomass collection

The two sampling campaigns (Sample 1 and 2) were done during steady state of the MBR and are shown in Fig. 2. First, bioreactor content was nitrogen-bubbled for 10 min to suspend biofilms and membrane-attached microorganisms. Afterwards, liquid aliquots containing the suspended biomass were taken separately from the upper and the bottom part of the bioreactor and mixed 1:1 v/v to a final 10 mL volume in duplicate samples. Samples were collected minimizing the oxygen exposure and frozen immediately at 20 °C. This whole procedure was repeated in three different time-points separated for at least three natural days for each of the two sampling campaigns. Proteins were extracted from each sample separately. The resulting 6 protein extracts for each sampling campaign were then pooled prior to MS/MS injection.

2.3.2. Protein extraction

The protocol for protein extraction was described in (Kennes-Veiga et al., 2022). Briefly, cells were centrifuged at 6000 rpm, washed twice with PBS buffer, digested at 90 °C, 20 min in 1% SDS Tris-HCl extraction buffer and broken by bead beating. Centrifugation at 3000 rpm was subsequently applied to remove cell debris and glass beads. Proteins

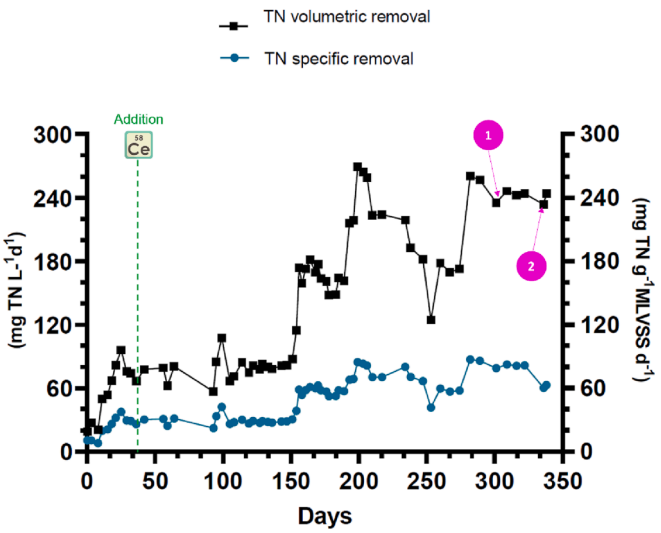

Fig. 2. TN in volumetric (by flow fed to the system) and specific (by biomass concentration) removal in the MBR. In circles, 1 and 2 correspond to both sampling campaigns for metal quantification and proteomic analyses. Nitrogen removal was 99–100 % during the period between sampling campaigns 1 and 2.

were then precipitated with acetone at −20 °C and resuspended in molecular grade water. Protein concentration was estimated by the BCA assay and the protein quality of the samples was checked by SDS-PAGE electrophoresis as detailed in (Kennes-Veiga et al, 2022).

2.3.3. Mass spectrometric analyses

Proteins were identified with a shotgun metaproteomic approach after "in-solution tryptic digestion" of the proteome samples (Zhang et al., 2013). For this, samples were reduced, alkylated, trypsin-digested, acidified, desalted, vacuum-dried and reconstituted in water with 2% acetonitrile (ACN) and 0.1% formic acid (FA) (Kennes-Veiga et al., 2022). Aliquots of the obtained peptide mixtures (200 ng) were analyzed in a nanoElute (Bruker) nano-flow liquid chromatograph (LC) equipped with a C-18 reversed-phase column coupled to a high-resolution TIMS-QTOF (timsTOF Pro, Bruker) with a CaptiveSpray ion source (Bruker). After ESI ionization, peptides were analyzed in data-dependent mode with the Parallel Accumulation–Serial Fragmentation (PASEF) enabled. These analyses were done at the Proteomics Unit of the CIBER-BBN at SERGAS (Spain) and the Centro Interdisciplinar de Química e Bioloxía (CICA) of the University of A Coruña (Spain) as detailed in Kennes-Veiga et al., 2022. The mass spectrometry proteomics data was deposited in the ProteomeXchange Consortium via the PRIDE[40] partner repository with the dataset identifier PXD040475 and https://doi.org/10.6019/PXD040475.

2.3.4. Protein data analyses

Mass spectrometry raw files were processed with PEAKS Studio 10.6 build 20,201,221 (Bioinformatics Solutions Inc.) in three different rounds of analyses. The detailed parameters for protein data computational analyses are presented in Text S2. First, data were interrogated against a self-constructed database containing all UniProtKB/Swiss-Prot (release 2022_03) protein sequences corresponding to *Methylomirabilis*. It contained a total of 3012 entries belong to the *M. lanthanidiphila*, 3125 to *M. oxyfera* and 2413 to *M limnetica*. Protein sequences corresponding to *M. sinica* were not found on available databases. The gene callings starting with MELA_from UniProtKB/Swiss-Prot database correspond to the genomic sequences from *M. lanthanidiphila* functionally annotated as described in Versantvoort et al., (2019) and Versantvoort et al. (2018). In the present study the protein names presented through the text were corrected according to this paper.

As a second approach, the authors applied a restricted reference

database, as described in Blakeley-Ruiz and Kleiner, (2022) including all available protein sequences belonging to the genera *Methylomirabilis, Hyphomicrobium, Denitratisoma, Ignavibacterium, Methylosinus, Rhodoplanes, Gaiella* and *Mehtylocystis* from the NCBI protein public database (September 2022). Those taxa were selected according to previous DNA metabarcoding sequencing of the 16S rRNA gene from the MBR bioreactor (data not shown). Last, a specific search against UniProt entries of the N-damo archaea *Methanoperedens* (Sept. 2022) was performed. The webtool Unipept Desktop 3.0. was used for analyses of the peptides identified on the MS/MS analyses.

3. Results and discussion

3.1. Reactor performance

The MBR system highly enriched with N-damo (60 % relative abundance of *Methylomirabilis*, according to 16S rRNA gene sequencing, Quiton-Tapia et al., 2023), operated for 346 days. The operation was characterized by a robust and stable reactor performance to reach and maintain the different high NLRs. During the MBR operation, the NLR was progressively increased from an initial concentration of 10 mg TN $L^{-1}d^{-1}$ to a maximum value of 269 mg TN $L^{-1}d^{-1}$ on day 199 (Fig. 2). The MBR operation sustained the increasing NLR with an efficient performance, as evidenced by the increase between day 147 and 156, from 88 to 176 mg TN L^{-1} d^{-1} in the TN volumetric removal. Sampling campaigns 1 and 2 were performed at the end of the operation at a stable NLR of 246 ± 5 mg TN $L^{-1}d^{-1}$ during a period of 35 days in between samples (corresponding to the operation days 300 till 335). The NLR accounted mainly for N-NO_2^-, which was the limiting substrate. Overall, the average nitrite concentrations in permeate was 0.9 mg N/L with a minimum value of 0.04 mg N/L (day 151) and a maximum value of 9.5 mg N/L (day 338). Traces of nitrogen as nitrate and ammonium were also detected in the feed, as the medium contained ammonium chloride (Arias et al., 2022) and possibly the sodium nitrite used as reagent contained some nitrate traces. Average nitrate removal rate was 2.9 mg N-NO3- L-1 d-1 with a minimum value of 0.04 (day 15) and a maximum value of 6.8 (day 95), while ammonium average removal rate was 1.9 mg N-NH4 + L- 1 d- 1, with a minimum value of 0 (day 15) and a maximum value of 3.6 (day 210). The nitrogen removal was maintained in the range of 72% (day 8) up to 100 % during the MBR operation, and between days 300 and 335 was between 99 - 100 %.

The pH was maintained in neutral range (average 7.8 ± 0.3) by modifying the inflow of the gas mixture (90 %CH_4/ 10 %CO_2). The average redox potential measurements were − 347.7 ± 135 mV. Methane was fed to the reactor in saturation, from a gas mix bottle 90/10 CH_4/CO_2 v/v and the gas flow was adjusted during the increase of NLRs to make the CO_2 counteract the alkaline production due to denitrification. Relatively stable gas composition on the headspace was maintained with values of 85.5% CH_4, 10.6 % N_2 and 3.8 % CO_2. As an indicator of system perturbances, N_2O production was continuously monitored. The N-damo metabolic pathway does not include N_2O as an intermediate and, accordingly, it was not present except in six specific days (day 0, 32, 154, 206, 217 and 234, below 0.01%) that coincided with reactor perturbances, causing temporary nitrite increases (below 9.5 mg N-NO_2− L^{-1}). N_2O production was attributed to heterotrophic denitrifying bacteria that probably reduced nitrite coupled to microbial decay products. Previous studies have also suggested that a temporal loss of N-damo activity caused by reactor perturbances can favor conventional denitrifying bacteria in N-damo enrichment cultures (Allegue et al., 2018; Arias et al., 2022; Quiton-Tapia et al., 2023).

TOC concentrations in the feed and permeate showed an average value of 3.7 ± 1.7 mg TOC L^{-1} and 4.6 ± 4.7 mg TOC L^{-1}, respectively (see supplementary material). The slight increase in the permeate TOC concentration was mainly due to four measurements with significantly higher TOC concentration in the permeate, on days 0, 105, 206, and 210, which ranged between 11.8 and 16.7 mg TOC L^{-1}. This increase could be attributed to biomass decay or by-products of methane oxidation. Recently, Guo et al, 2023 demonstrated the methane-derived production of formate by *Ca.* Methylomirabilis which was used on their study by heterotrophic denitrifiers for denitrification and biomass growth, providing new insights into the syntrophic interactions in N-damo systems. It is, therefore, a potential explanation that the formate non assimilated by the biomass trespassed the membrane and reached the permeate.

3.2. Metal consumption

The trace metal concentration was maintained in the feed during the whole reactor operation. Cerium was added in the mineral feed on day 33 onwards in a constant concentration of 4.5 μg L^{-1} and it was completely consumed across the operation. The other metals were consumed to different extents (Fig. 3). In the case of Fe, Cu and Zn an appreciable decrease on their concentration was observed (>10%) while other metals like Mn, Se, Co, W, Ni and Mo did not show an appreciable difference (<10%) between the feed and permeate concentrations. The addition of a chelating agent (EDTA) has been demonstrated to improve metal bioavailability, specially concerning Fe, Cu y Zn in both the feed and the permeate (Arias et al, 2022) although in the referred study, cerium was not included in the feed composition.

3.3. Methylomirabilis proteome expression

3.3.1. Strains differential abundance

The six protein extracts corresponding to each sampling campaign on the bioreactor were obtained and pooled prior its nLC-MS/MS injection. The protein concentrations and SDS-gel profiles of each sample are presented in (see supplementary material). A total of 1262 and 1365 proteins belonging to the genus *Methylomirabilis* were identified in PEAKS analysis and 567 and 651 of them with 2 or more unique peptides in samples 1 and 2, respectively. The aminoacid sequences of all peptides detected in this analysis (over 1500 per sample) were cleaned and subsequently analyzed by Unipept 3.0. for treeview visualization of the taxonomies (Fig. 4). Our proteomic results indicate a strong predominance of *M. lanthanidiphila* over the other two *Methylomirabilis* species at the two sampling campaigns (Fig. 4), which can be attributed to the operational conditions, specially to cerium supplementation, and not the number of sequences from each of them available in the database used in this study.

3.3.2. Protein functional categorization

The percentage of peptides belonging to each Enzyme Commission and Gene Onthology (molecular function) category was calculated with Unipept 3.0. and is presented in (see supplementary material). Regarding EC classification the most abundantly expressed category is

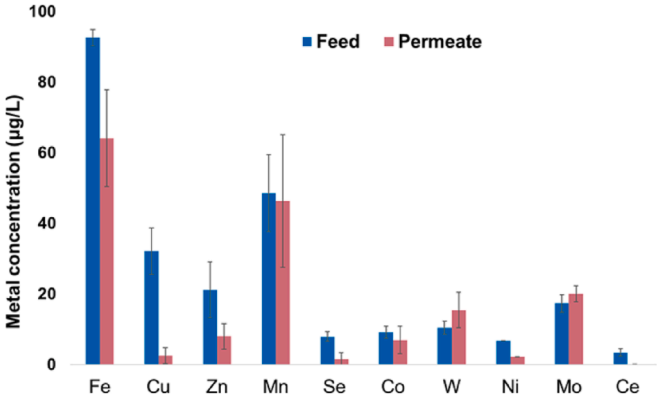

Fig. 3. Trace metal concentrations in the feed and permeate of the MBR (n = 3).

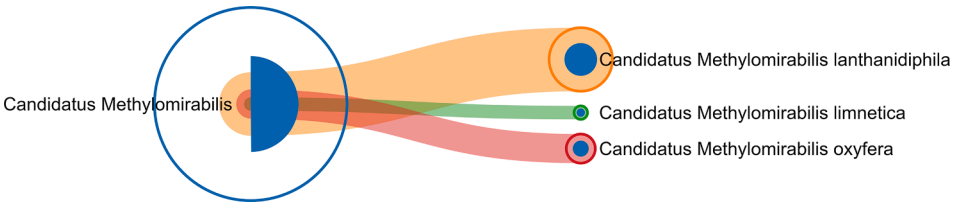

Fig. 4. Treeview visualization of the relative abundance of each *Methylomirabilis* strain on the N-damo enriched MBR reactor metaproteome during steady state (on days 300 and 335) at high-nitrogen loading rate (246 ± 5 mg TN L^{-1} d^{-1}).

DNA-directed RNA polymerases followed by metalloendopeptidases and RuBisCo, which is the major player on carbon fixation from CO_2 into organic biomass via the Calvin–Benson–Basham pathway. Nitrite reductase and methane monooxygenase activities are found at the 7th and 18th position, respectively. Regarding Gene Ontology, the most abundant categories were ATP binding, metal ion binding and DNA binding. In both categorizations it is clear how the variety and availability of metals in the environment is crucial for bacterial fitness and is reflected at the proteome level. In this sense, categories metal ion binding, magnesium ion binding, zinc ion binding, heme binding, metalloendopeptidases, iron-sulfur cluster binding, calcium ion binding and molybdenum cofactor binding are among the most abundant ones (see supplementary material).

3.3.3. Methane oxidation

Methylomirabilis first oxidize methane to methanol by the copper-dependent membrane-bound enzyme: particulate methane monooxygenase (pMMO), which consists of three subunits. The PmoA and PmoC cross the membrane while PmoB has two transmembrane helices and a soluble β-barrel motif which forms a hole for substrate entry and product exit (Ross et al., 2019). The three subunits were detected in our bioreactor samples (see supplementary material). The *Methylomirabilis* genomes described so far do not encode for the counterpart soluble methane monooxygenases (sMMO), in contrast to most methanotrophs (Versantvoort et al., 2018).

The next step in this route is methanol oxidation to formaldehyde. There are two known groups of methanol dehydrogenases (MDH), the canonical MxaFI type, which carries calcium as essential cofactor and the more recently discovered XoxF-type, which binds lanthanides in the active site (Keltjens et al., 2014). The most abundantly expressed MDH from *Methylomirabilis* in our bioreactor belongs to the XoXF type and specifically to *M. lanthanidiphila (*MELA_00916). The other one detected belong to the MxaF type and to *M. oxyfera* (D5MI98|D5MI98_9BACT). Interestingly, regarding the sequence analyses described in Versantvoort et al., (2018), the genome of *M. lanthanidiphila* encodes for a single XoxF type MDH while the cluster encoding for the calcium-MDH is absent. In contrast, *M. oxyfera* genome is redundant encoding for canonical MxaFI MDHs and a XoxF type one (Wu et al., 2015). This difference relates with the advantage of *M. lanthanidiphila* over *M. oxyfera* when growing in the presence of cerium. A specific aspartate residue was found to play a crucial role in the rare-earth element interaction with the active site of Xox type MDH (Keltjens et al., 2014). From genomic analyses '*Ca. M. limnetica*' is also most likely dependent on lanthanides to produce an active MDH, yet in our experiments this strain was detected in a much lower relative abundance than the other two. The reason for this difference remains unknown to us.

Formaldehyde can be oxidized to formate by XoxF-MDH, but *Methylomirabilis* species also encode for two specific systems to oxidize it. In the first one the 5,6,7,8-tetra-hydromethanopterin pathway (H_4MPT) act as the C1 carrier, while in the second, this role is taken by the 5,6,7,8-tetrahydrofolate (H_4F). Formaldehyde activating enzyme, methylene-H4MPT dehydrogenase and methenyl-dH4MPT cyclohydrolase are the three enzymes responsible for the sequential formation of 5-formyl-H_4MPT from formaldehyde in the first described route and were accordingly found expressed. In the H_4F pathway, a methenyl-H_4F-cyclohydrolase and formyl-H4F lyase are the actors and were also found expressed. The subsequent formate oxidation system consisting of FdhA and FdhB. In our study, the FdhA subunit was detected. In FDHs, the cofactor is coordinated to either molybdenum (Mo-bisPGD) or tungsten (W-bisPGD) at which the metal additionally binds either cysteine or selenocysteine (Reimann et al., 2015).

Methylomirabilis are autotrophic, and in contrast to other methanotrophs fix carbon by the Calvin-Benson-Bassham (CBB) cycle instead of the serine pathway or ribulose monophosphate (RuMP) cycle (Ettwig et al., 2010). The main step in this pathway is catalyzed by the RuBisCo enzyme and consists of the carboxylation of ribulose-1,5-bisphosphate. From all RuBisCo types found in nature, the one encoded by *Methylomirabilis* belongs to type I (Versantvoort et al., 2019).

In regards of electron transfer, *M. lanthanidiphila* encodes for multiple cytochrome bc1 complexes, which is not uncommon in nature, as these complexes can be inserted into any type of respiratory chain mediated by quinones. Accordingly, peptides belonging to cytochrome biogenesis proteins were detected in the present analyses (i.e: cytochrome *c*-type biogenesis protein CycK, CcmF and CcmE).

3.3.4. Metabolism of nitrogenous compounds

N-damo bacteria reduce nitrite into nitric oxide by a cytochrome cd1 type nitrite reductase (Cd1-NIR), using copper as metal cofactor. The next step is performed by their unique NO dismutase (No-d) and consists of the dismutation of two molecules of nitric oxide into molecular oxygen and nitrogen gas. As detailed in Versantvort, et al, 2019, this reaction might be the limiting step in *Methylomirabilis* metabolism, though it its thermodynamically feasible. No-d belong to the group of nitric oxide reductases but have undergone amino acid substitutions in their catalytic sites, quinol binding sites and proton channels that impede NO reduction to N_2O (Ettwig et al, 2012). Additionally, *Methylomirabilis* encode canonical quinol-dependent nitric oxide reductases (NOR), preventing NO accumulation, that was also found expressed. NOR are also electrogenic enzymes, transferring electrons from the ferrous irons for NO conversion coupled to ATP production.

N-damo bacteria encode for a single F_1F_0 ATP synthase to harvest the proton-motive force (pmf) produced by the various respiratory complexes and form ATP. Several ATPase subunits were detected in our shotgun analyses: specifically: α, β, γ, B and C.

In this work, by in solution shotgun analyses it was possible to identify all enzymes involved in the N and C cycle from *Methylomirabilis* and their corresponding peptide numbers and relative abundances are shown in (see supplementary material). The full list of identifications can be found in Supplementary excel file. A schematic representation of the enzymatic pathway for the simultaneous nitrite reduction and oxygen-dependent methane oxidation "*Ca.* Methylomirabilis", the number of peptides identified for each enzyme and their corresponding metal cofactor is shown in Fig. 5.

3.3.5. Other physiological features from Methylomirabilis

Metaproteomics allowed us to detect other aspects from *Methylomirabilis* lifestyle not directly linked with the removal of target pollutants, but with its general physiology. Regarding oxidative stress defense, three iron-containing superoxide dismutases (MELA_02229 from *M. lanthanidiphila* and DAMO_0395 from *M. oxyfera* and

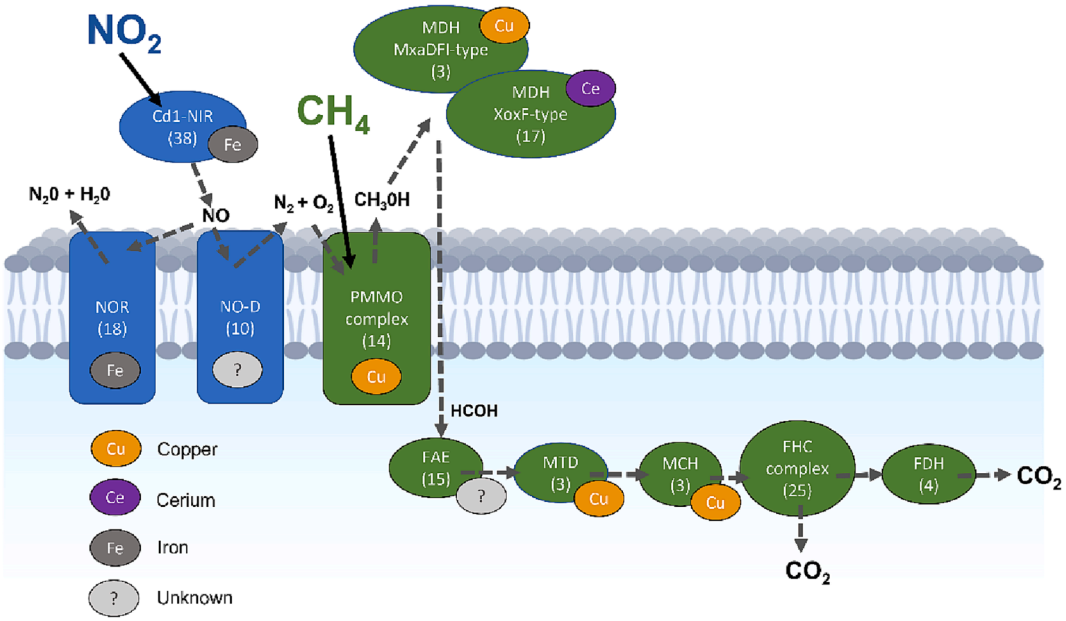

Fig. 5. Scheme of the anaerobic metabolism of N-damo bacteria ("*Ca.* Methylomirabilis") showing the enzymatic routes for nitrite reduction and subsequent dismutation of NO into N_2 and O_2 by their unique NO dismutases. The generated oxygen is used for methane oxidation initiated by their methane monooxygenases. Enzymes from the nitrite reduction route are represented in blue colour while those from the methane activation route are represented in green. For each enzyme we show the number of total peptides (in brackets) detected in this study and their corresponding metal cofactor (in circles). Adapted from the model described in (Versantvoort et al., 2019).

CLG94_07065 from *M. limnetica)* were expressed. The authors speculate they serve as protection mechanism in case of transitional periods of oxidative stress if intracellular oxygen from Nod activity is not consumed, e.g., in methane or copper deficit. Methane-utilizing bacteria typically flourish in nature at the aerobic-anaerobic interface, as they require the oxygen for the methane activation, so from a biological point of view they should have a defence against oxidative stress. Interestingly, the expression of the *M. oxyfera* superoxide dismutase (DAMO_0395) was also detected in Hanson and Madsen, (2015), in environmental subsurface waters. Superoxide dismutases have also been shown to have a role in biofilm formation and bacterial morphology in other anaerobes (Najmuldeen et al., 2019).

Twitching motility plays a key role in bacterial biofilm formation and was confirmed by the detection of the twitching motility protein (PilT) and Type IV pilus assembly protein (TapB) (Cursino et al., 2009). Interestingly, the cell shape-determining protein MreB, which was often associated to non-spherical shape bacteria in previous literature was detected (White and Gober, 2012). However, mela_0085 which was found in Gambelli et al., (2021), as the main S-layer protein from *M. lanthanidiphila* and involved in their cell shape was not detected in our study.

Considering metal intake and its transport, the following proteins were also expressed: TonB-denpendent receptor, TonB-dependent outer membrane receptor for cobalamin and iron transport, iron-sulfur cluster carrier protein, putative heavy metal efflux pump CzcB family, molybdopterin molybdenum transferase, magnesium, and cobalt efflux protein CorC, putative molybdopterin biosynthesis protein moeB (modular protein) and molybdenum cofactor biosynthesis protein B.

3.4. Activities of the other members of the microbial community

To investigate the individual proteins related to metabolic functions active on the predominant bacterial genera present in the MBR a second metaproteomic analysis using a restricted reference database, based on previous 16S rRNA sequencing analyses was performed (See Fig. 6). House-keeping related proteins (e.g., ribosomal related proteins, chaperons and chaperonins or elongation factors) were therefore not considered (see supplementary material).

The high relative abundance of cytochrome cd1-type nitrite reductases identified from *Denitratisoma* indicate how members of this genus were playing the role of denitrifiers in our bioreactor and probably competing with *Methylomirabilis* for this main substrate. In previous studies this genus was also found to transform nitrite in simultaneous anammox denitrification (SAD) bioreactors and to be an endogenous denitrifier, specifically degrading the decaying bacteria usually existing in annamox enrichments (Ma et al., 2017; Wang et al., 2019).

Ignavibacterium was involved in denitrification, as indicated by the detection of the Sec-dependent nitrous oxide reductase catalyzing the final step of this route, the reduction of nitrous oxide to dinitrogen and activating the acetate by the acetate CoA ligase. Even though members of *Ignavibacterium (i.e: I. album)* harbor genes encoding respiratory nitrite reductases and nitric oxide reductases (Liu et al., 2012), those were not detected.

Both *Methylosinus* and *Methylocystis* were very active in oxidizing methane in regards of their abundantly expressed methane monooxygenases pMMO and periplasmic pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenases (calcium-containing). This is in line with recent literature demonstrating that aerobic methanotrophs and specifically these two genera can couple the oxidation of methane with reduction of metals, nitrate, nitrite, or nitrous oxide in the absence of oxygen (Cheng et al, 2022). It would deserve more research effort to decipher the electron acceptor/s used by these methanotrophs in the N-damo system.

Last, methylotrophy is the main activity detected for *Hyphomicrobium*, regarding its expression of (PQQ)-dependent methanol dehydrogenases, 5–6-7–8-tetrahydromethanopterin-hydrolyase and formaldehyde-activating enzyme (FAD). *Rhodoplanes* role in the community could not be clarified as chaperonins, elongation factors and adenosyl homocysteinase were the only proteins detected.

3.5. Nitrate consumption

The trace amounts of nitrate detected in the influent were consumed along the operation of the bioreactor resulting in a removal rate of 3 ±

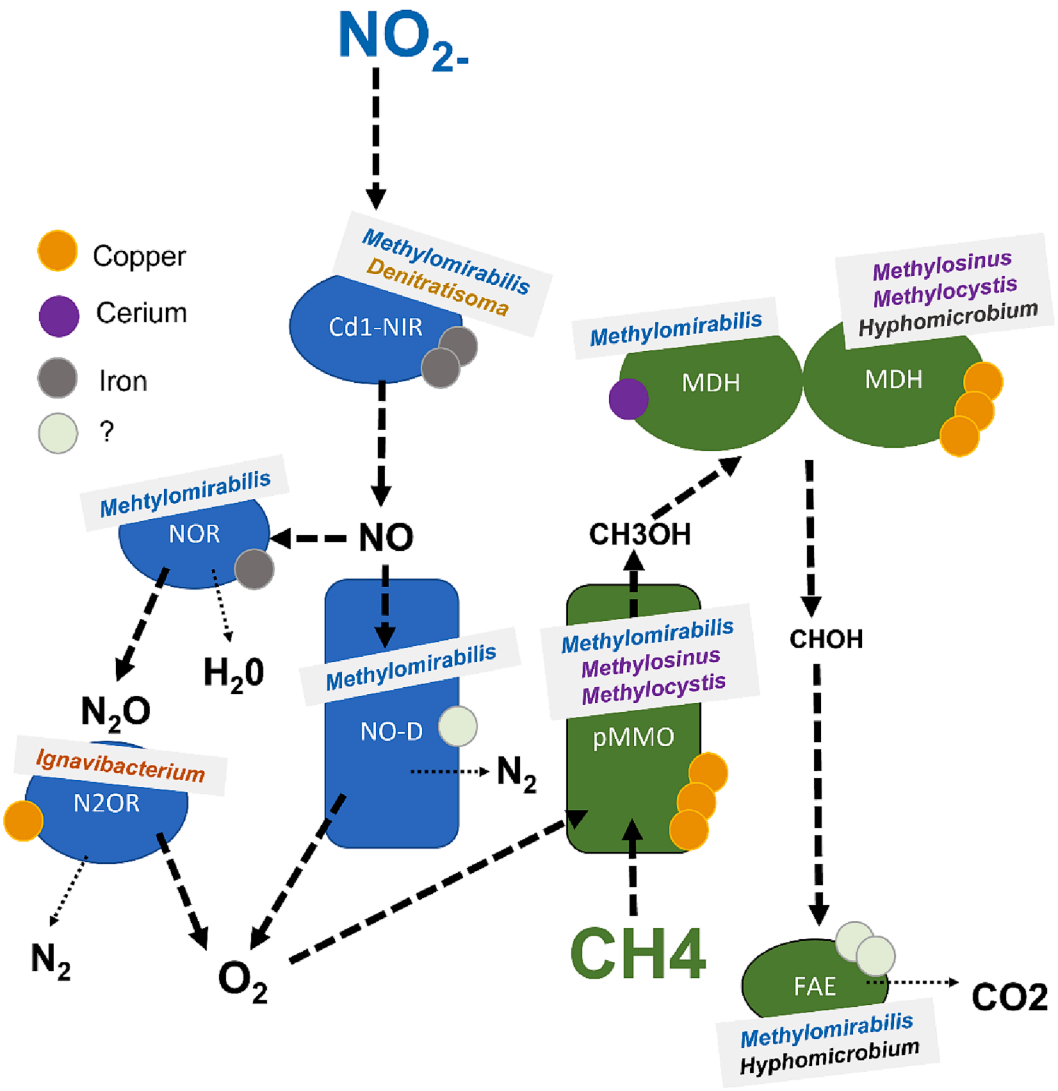

Fig. 6. Metalloenzymes from the members of the N-damo enriched MBR community involved in the nitrogen and methane cycle according to metaproteomic analyses.

1.4 mg N-NO_3^- L^{-1} d^{-1}. N-damo archaea (*Ca.* Methanoperedens) have been previously shown to reduce nitrate to nitrite (Haroon et al., 2013) and to investigate its presence and potential nitrate consumption a specific search for its proteins in a third round of analyses was included. The corresponding identifications are presented in (see supplementary material). Proteins with at least 2 total peptides detected were considered, although the sequence coverage was very low (below 10%), regarding the interest on N-damo archaea for this study. The low number of identifications (4) manifests the low abundance of *Methanoperedens* in the community. The most abundantly detected protein was an ATP dependent chaperone (ClpB) followed by the nitrogen regulatory protein P-II (one of the most widely distributed families of signal transductors in nature) and a DNA-directed RNA polymerase subunit. ClpB is a disaggregase known to inhibit and reverse protein aggregation during stress survival (upon reactive oxidative species, pH and osmolarity changes) but also during normal physiological conditions (Alam et al., 2021).

Interestingly, the nitrate reductases identified in this study belong to *Methylomirabilis* and not to *Methanoperedens, Hyphomicrobium or Ignavibacterium* (also harboring nitrate reductases on their genomes). These were specifically the nitrate reductase catalytic subunit, Nar (identified with 9 peptides), periplasmic nitrate reductase subunit alpha, NapA (identified with 5 peptides) and nitrate reductase subunit beta, NapB (identified with 2 peptides). So far it couldn't be predicted from sequence analyses neither by biochemical evidence whether the nitrate reductases from *Methylomirabilis* function as nitrite-oxidizing or nitrate-reducing protein complexes. Previous enrichment cultures containing *Methylomirabilis* only fed with nitrite (no nitrate) were found to be expressing the three systems to reduce nitrate to nitrite that its genome harbor (Versantvoort et al., 2019).

4. Conclusions

Iron and copper were among the most consumed metals, as they are cofactors for the enzymes: nitrite reductases, nitric oxide reductases, MDH and pMMO. The predominance of "*Ca.* Methylomirabilis lanthanidiphila" over other "*Ca.* Methylomirabilis" strains is attributed to the addition of cerium inducing the expression of its Xox-F-type MDH with affinity with lanthanides. To promote N-damo activity, the metal composition in the feed could be enhanced by increasing concentrations of cerium and copper, as these metals were almost fully consumed. This study exemplifies the usefulness of metaproteomics to help on decision making towards promoting preferred microbial activities during biotechnological processes.

CRediT authorship contribution statement

Silvana Quiton-Tapia: Methodology, Investigation, Data curation, Writing – review & editing. **Alba Trueba-Santiso:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Juan M. Garrido:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition. **Sonia Suarez:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition. **Francisco Omil:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Supplementary data was included in two supplementary files

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129476.

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