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Operating strategies to optimize a membrane bioreactor enriched in nitrite-dependent anaerobic methane-oxidizing bacteria

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ABSTRACT

The use of *N*-damo bacteria, which can simultaneously remove nitrite and dissolved methane, could represent a cost-effective and sustainable alternative to minimize the environmental impact of effluents from methanogenic reactors treating domestic sewage. The operation of *N*-damo bacteria reactors is often not stable, and their activity decrease with no proven reason over time. This study aims to optimize the nitrite removal capacity of a lab-scale MBR by using different operating strategies for 878 days. The reactor was operated in continuous mode and with biomass highly enriched in these microbes. It was found that adjustments in the mineral medium concentration (reduction of Ca, P, and K) and composition (EDTA addition, increasing the Cu and Fe bioavailability), contributed to achieving remarkable and prolonged nitrite removal rates of up to 285.7 mg NO_2^--N/L/d. *N*-damo bacteria dominated the culture, up to 57 %. To the best of our knowledge, the rates attained are the highest reported in an *N*-damo bacteria enrichment.

1. Introduction

A potential strategy to mitigate the main environmental impacts of effluents from methanogenic reactors treating domestic sewage, such as methane (strong greenhouse gas) emissions and eutrophication, could consist of using bioprocesses in which methane is used as an inexpensive electron donor to remove nitrogen [1]. In this scenario, bacteria affiliated with the candidate NC10 phylum, such as *Candidatus* "Methylomirabilis oxyfera" [2], *Candidatus* "Methylomirabilis sinica" [3], and *Candidatus* "Methylomirabilis lanthanidiphila" [4], can oxidize methane by using nitrite as an electron acceptor in anaerobic conditions (Eq. 1). These microorganisms are collectively called nitrite-dependent anaerobic methane-oxidizing (*N*-damo) bacteria. They are found in natural habitats such as freshwater sediments [5], freshwater wetlands [6], oligotrophic freshwater lakes [7], minerotrophic peatlands [8], saline water environments [9], and also in the sludge of sewage treatment plants (STPs) [10].

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad (1)$$

$$NH_4^+ + 1.32NO_2^- \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O \quad (2)$$

$$3CH_4 + 3O_2 + 4NO_2^- + H^+ \rightarrow 2N_2 + 8H_2O + 3CO_2 \quad (3)$$

The use of post-treatment systems with *N*-damo bacteria to treat effluents of methanogenic reactors could result in great interest in future STPs for the following reasons: the addition of costly external carbon sources such as methanol or ethanol, are not required to remove nitrogen in a further post-treatment system; complete removal of nitrogen from sewage might be feasible; complete nitrification is not needed, which could result in energy savings; *N*-damo bacteria are autotrophic

Abbreviations: AMO-D, aerobic methane oxidation coupled to denitrification; CSTR, continuous stirred tank reactor; EDTA, ethylenediamine tetraacetic acid; HRT, hydraulic retention time; ICP-MS, inductively coupled plasma mass spectrometry; MBfR, membrane biofilm reactor; MBR, membrane bioreactor; MSGLR, magnetically stirred gas lift reactor; MLTSS, mixed-liquor total suspended solids; MLVSS, mixed-liquor volatile suspended solids; *N*-damo bacteria, nitrite-dependent anaerobic methane-oxidizing bacteria; NRR, nitrogen removal rate; OTUs, operational taxonomic units; SBR, sequencing batch reactor; SEM-EDX, scanning electron microscopy-energy dispersive X-ray spectroscopy; STP, sewage treatment plant; TN, total nitrogen.

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microorganisms with less sludge production than conventional anoxic–oxic wastewater treatment; this process is stoichiometrically more advantageous compared to aerobic methane oxidation coupled to denitrification. Despite all these advantages, *N*-damo bacteria processes are currently in their infancy and still far from being implemented on large scale. Furthermore, they have been studied only at bench-scale and under very controlled conditions.

In the last years, several studies of *N*-damo bacteria reactors have been carried out. Kampman et al. [11] operated a sequencing batch reactor (SBR) and achieved a nitrogen removal rate (NRR) of 38 mg NO_2^--N L^{-1} d^{-1}. Other studies using SBR configurations reported NRRs of 40 mg NO_2^--N L^{-1} d^{-1} [12] and 33 mg NO_2^--N L^{-1} d^{-1} [13]. Biomass washout, together with the slow doubling time of 1–2 weeks [2], and the low reported activities, indicate the need for further research.

Hu et al. [6] studied the impact of several reactor configurations in three different *N*-damo bacteria enrichments operated at identical conditions (30 °C; HRT 2 d): continuously stirred tank reactor (CSTR), SBR, and a magnetically stirred gas lift reactor (MSGLR). After 75 days of operation, maximum NRRs of 26.4, 11.4, and 76.9 mg NO_2^--N L^{-1} d^{-1} were achieved, respectively. It was suggested that the higher nitrite removals observed in the MSGLR were the result of an improvement in the gas (CH_4)-liquid mass transfer, which could be crucial for optimum *N*-damo performance. However, a significant biomass washout was observed. To avoid the washout and completely control the biomass retention, Kampman et al. [14] operated a lab-scale MBR for more than 1,000 d, and despite guaranteeing complete biomass retention, a decrease in the nitrite consumption was observed after reaching a maximum NRR of 36 mg NO_2^--N L^{-1} d^{-1}. The reason for the drop in activity was not elucidated. In another MBR system, Allegue et al. [15] reported higher apparent nitrite removals rates, with values of 103 mg NO_2^--N L^{-1} d^{-1}. Lower HRT (1 d) and higher temperatures (28°C) were applied compared to Kampman et al. [14]. Relative abundances for *N*-damo bacteria of 50.2 % were attained and less than 100 days were needed to obtain those activities.

Overall, the operation of *N*-damo bacteria reactors is reported to be frequently unstable, and their activity decrease over time with no proven reason. Besides, nitrite removal rates above 103 mg NO_2^--N L^{-1} d^{-1} have not been reported yet. This study aims to optimize the nitrite removal potential of a lab-scale MBR system operated in continuous mode and with suspended biomass enriched in *N*-damo bacteria. High and prolonged activities are intended. For this purpose, different operating strategies were followed such as reducing the macronutrients (Ca, K and P) concentration in the feed, and improving the bioavailability of trace elements (Fe and Cu) by using EDTA. Besides, the impact of dissolved oxygen and diluted seawater on the enrichment activity is also evaluated.

2. Materials and methods

2.1. Reactor configuration and operation

In the present study, the operation of a lab-scale MBR system of 10 L (working volume 6.65 L), previously ran for 388 days by Allegue et al. [15], has been continued. That last operating day on that previous study (Day 388) is considered Day 0 in the current one. The sludge at the end of the previous study was mostly enriched in *N*-damo bacteria (50.2 %) and presented an apparent volumetric nitrite removal rate of 103 mg NO_2^--N L^{-1} d^{-1} [15]. The mixed-liquor volatile suspended solids concentration (MLVSS) inside the reactor was 1.1 g/L.

A scheme of the operating system is depicted in Fig. 1. Inside the reactor, a 0.5 m^2 hollow-fiber submerged ultrafiltration membrane (Puron) (Fig. S.1), with 0.03 μm of pore size, was used to allow complete biomass retention. Membrane operating cycles were 7 min of permeation and 30 s of relaxation. A pressure sensor PN2069 (IFM) indicated the permeation and relaxation pressures. A fixed permeate flow of 7 L d^{-1} was applied. Besides, the temperature was maintained at 28 °C.

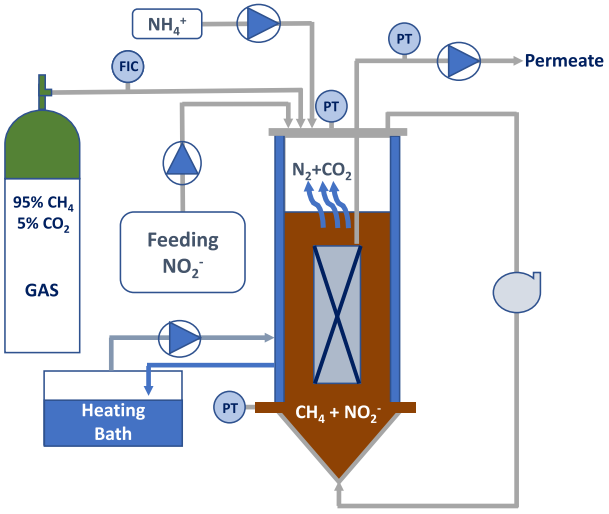

Fig. 1. Scheme of the MBR system. Where FIC is a flow indicator controller, PT is a pressure transmitter and FIT is a flow indicator transmitter. Between days 12–249 and 341–766, the ammonium was fed in batch mode by using a peristaltic pump, meanwhile, ammonium was fed in continuous mode during the remaining periods.

The mixing liquor was continuously gasified with a gas mixture (5 % CO_2 and 95 % CH_4), by using a mass-flow controller E-7000 (Bronckhorst). CH_4 and CO_2 were added in excess at the top of the reactor to avoid any buffer or electron donor limitation during the experimentation period, respectively. Both, the CO_2 and the sodium bicarbonate, provided enough buffer capacity to the system since *N*-damo bacteria reaction alkalinizes the medium (Eq. 1). The gas flow varied from 5.4 to 24.5 L d^{-1}, depending on the MBR activity, to counteract the pH increases. The higher the nitrite removal the higher the gas flow applied. A mini laboratory blower N 86 KT 18 (Laboport) was used to recirculate 5.5 L min^{-1} of gas from the top to the bottom of the reactor to prevent membrane fouling, keep a homogeneous mixing, and maximize methane availability. The operation of the system was controlled by a programmable PLC Micro 820 (Allen-Bradley) connected to a computer. Dissolved oxygen was desorbed from the feeding aluminum foil bags (totally impermeable to oxygen), during most of the operation (but during PII), by diffusing nitrogen gas carefully for at least 10 min. During PII, the feeding bags were gasified with air, to evaluate the effect of dissolved oxygen on the reactor performance.

The following synthetic medium was used as the substrate for the MBR (per liter of feed): 100 mg $NaHCO_3$; 100–1,232 mg $NaNO_2$, depending on the nitrite removal activity of the system; 14.4–24.8 mg of NH_4Cl. Ammonium was added to the feed as an extra nitrogen assimilation source, besides nitrite; the same macronutrients composition as proposed by Allegue et al. [15]: 50 mg KH_2PO_4, 200 mg $CaCl_2$, and 10 mg $MgSO_4$; the trace elements were added as suggested by Ettwig et al. [16]: 0.5 mL of an acidic and 0.2 mL of an alkaline trace elements solution. The acidic (100 mM HCl) trace element solution contained (per liter): 2.085 g $FeSO_4{\cdot}7H_2O$, 0.068 g $ZnSO_4{\cdot}7H_2O$, 0.12 g $CoCl_2{\cdot}6H_2O$, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.32 g $CuSO_4$, 0.095 g $NiCl_2{\cdot}6H_2O$, and 0.014 g H_3BO_3. The alkaline (10 mM NaOH) trace elements solution contained (per liter): 0.067 g SeO_2, 0.050 g $Na_2WO_4{\cdot}2H_2O$, and 0.242 g Na_2MoO_4. The macronutrients and trace elements composition in the feed has been changed during the study.

2.2. Operating strategies

Six main operating periods can be distinguished: PI (days 0–81) was characterized by a significant drop in the denitrification potential of the MBR. Same experimental conditions as Allegue et al. (2018) were applied; PII (days 82–249), the impact of dissolved oxygen on the *N*-

damo bacteria activity was tested; PIII (days 250–396), the same operating conditions as in PI were provided; PIV (days 397–716), highly diluted seawater was added in the feed line as an extra source of ions (nutrients). It was intended to test if some of the nutrients present in the seawater could improve the denitrification potential; d) PV (days 718–781), lower Ca, P, and K concentrations in feed were added to avoid salts precipitation, which could negatively affect to the reactor performance. Besides, biofilm attached to the reactor walls and the membrane's fibers were resuspended; PVI (days 782–878), EDTA was added to the mineral medium to improve the bioavailability of trace elements such as copper (Cu) and iron (Fe) for *N*-damo bacteria. The main characteristics of each period are resumed in Table 1.

2.3. Analytical methods

Gas and liquid samples were taken from the reactor 2–3 times per week. Temperature, pH, nitrite, nitrate, ammonia, mixed-liquor total suspended solids (MLTSS), and MLVSS, were determined according to Standard and Methods [17]. The dissolved oxygen in the feeding bags was analyzed by using a multi-parameter meter (Hach HQ40d), with a luminescent optical probe (IntelliCAL LDO101). A MilliGascounter (MGC-1 V3.3 PMMA, Ritter) was employed to determine the gas flow added to the MBR. The gas-phase composition was determined using a gas chromatograph HP 5890 Series II with a Porapack Q 80/100 2 m × 1/8″ (SUPELCO) column. The oven temperature (column) was set at 35 °C; meanwhile, the injector and the detector temperatures were set both at 110° C. Trace elements composition in the feed and effluent were measured by inductively coupled plasma mass spectrometry (ICP-MS).

2.4. Microbial diversity determination by using 16S rRNA gene amplicon sequencing

The microbial communities of the MBR system were studied on 9 different operating days by using 16S rRNA gene amplicon sequencing. The biomass samples were directly withdrawn from the bioreactor and immediately poured into previously sterilized 1.5 mL Eppendorf tubes. Later, these tubes were stored immediately in the freezer at temperatures below 20 °C. The total genomic DNA was extracted using the Stool DNA Isolation KIT (Norgen. Thorold, Canada). Total DNA concentrations were quantified in a Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA) and checked for size integrity by standard electrophoresis.

The V3V4 hypervariable region for Bacteria was amplified using Bakt_341F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) [18]. DNA metabarcoding analyses of the region were carried out by AllGenetics & Biology SL. Bioinformatic analysis of NGS (Next Generation Sequencing) data was performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). Raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTUs) at a 97 % cutoff for sequence similarity and classified against the non-redundant version of the SILVA SSU reference taxonomy database [19].

Table 1
Description of the different operating periods.

Operating Period	Operating days	Comments
I	0–81	Drop in the nitrite removal rates
II	82–249	Presence of dissolved O_2 in the feed
III	250–396	Same operating conditions as in PI
IV (a)	397–542	Addition of filtered and highly diluted seawater (1:20 v/v)
IV (b)	591–717	Addition of filtered and highly diluted seawater (1:100 v/v)
V	718–781	Lower concentrations of Ca, K, and P in the mineral medium; and biofilm attached to reactor walls and the hollow fibers of the membrane was resuspended (day 745)
VI	782–878	Addition of EDTA in the mineral medium to improve trace elements bioavailability

3. Results and discussion

The MBR was continuously operated for 878 days, in which the impact of different operational strategies on the nitrite removal capacity of the system was evaluated. The system was run at 28.2 ± 0.8 °C, with a fixed permeate flow of 6.96 ± 0.40 L d^{-1}, indicating a hydraulic retention time (HRT) of 0.96 ± 0.05 d. The nitrite loading rate was modified according to the denitrification activity, maintaining low nitrite concentration values in the permeate, between 0 and 39 mg N L^{-1}, to avoid possible inhibition events [20]. The nitrite concentration in the feed ranged between 9.5 and 277.4 mg NO_2^--N L_{Feed}^{-1} (Fig. 2). The pH varied from 6.35 to 8.44 with an average of 7.66 ± 0.33.

3.1. Drop in the denitrification capacity of N-damo bacteria

During the first 7 experimental days, the MBR presented high volumetric nitrite removal rates, 116 mg NO_2^--N L^{-1} d^{-1}, continuing with the values previously accomplished by Allegue et al. [15]. However, from that day 7, the activity gradually decreased, and only 32 mg NO_2^--N L^{-1} d^{-1} was achieved on day 16 (Fig. 2). This drop was concomitant with the absence of ammonium in the mixed liquor (Fig. S2). It was initially hypothesized that the lack of ammonium could result in lower activities. Besides nitrite, ammonium has been added to the feed, as an extra nitrogen assimilation source to avoid any possible growth limitations [21,22]. Ammonium was added at low concentrations, 8 mg NH_4^+-N L_{Feed}^{-1}, to limit the growth of anammox (Eq. 2) since these microbes, due to their higher affinity for nitrite, could outcompete *N*-damo bacteria [13].

To ensure the bioavailability of ammonium, spikes of ammonium (30 mL) were daily added to the MBR (between days 12 and 249) by using a peristaltic pump. Once added, an initial concentration of 8 mg NH_4^+-N L^{-1} was measured in the MBR. By doing so, the ammonium presence was guaranteed in the system for at least 5 h per day. Nevertheless, this strategy did not have any apparent effect on the nitrite removal rates, which remained stable and low from day 12 until the end of PI, with an average value of 29.5 ± 3.9 mg NO_2-N L^{-1} d^{-1}. This represents a drop of around 75 % compared to the first 7 operating days. Moreover, by using stoichiometric analysis and considering that all the observed ammonium consumption (8.3 mg NH_4^+-N L^{-1} d^{-1}) was conducted by anammox bacteria (Eq. 2), an apparent activity of only 11 mg NO_2^--N L^{-1} d^{-1} could be estimated for *N*-damo bacteria.

3.2. Impact of dissolved oxygen on N-damo bacteria

During PII (days 83–249) the impact of dissolved oxygen on the *N*-damo bacteria activity was evaluated. The objective of this approach was to verify if traces of this compound could favor or not, the development and stability of the enrichment. For this purpose, the feeding bag was gasified with air until saturation. The average oxygen concentration measured in the feed during this period was 8.3 ± 3.0 mg O_2 L^{-1}. Once dissolved oxygen was added, the activity of the reactor slightly but immediately increased reaching 77 mg NO_2^--N L^{-1} d^{-1} (day 147), doubling the value achieved by the end of PI, 29.3 ± 3.9 mg NO_2^--N L^{-1} d^{-1}.

A fraction of the dissolved oxygen could have been used either in ammonia and/or nitrite oxidation processes or by aerobic methane oxidation coupled to denitrification (AMO-D). Ammonia-oxidizing bacteria of the *Nitrosomonadaceae* family, and nitrite-oxidizing bacteria of the *Nitrospiraceae* family, were not affected by the presence of dissolved oxygen in feed, with abundances lower than 0.1 % during the entire experimentation period. In the AMO-D, aerobic methanotrophs

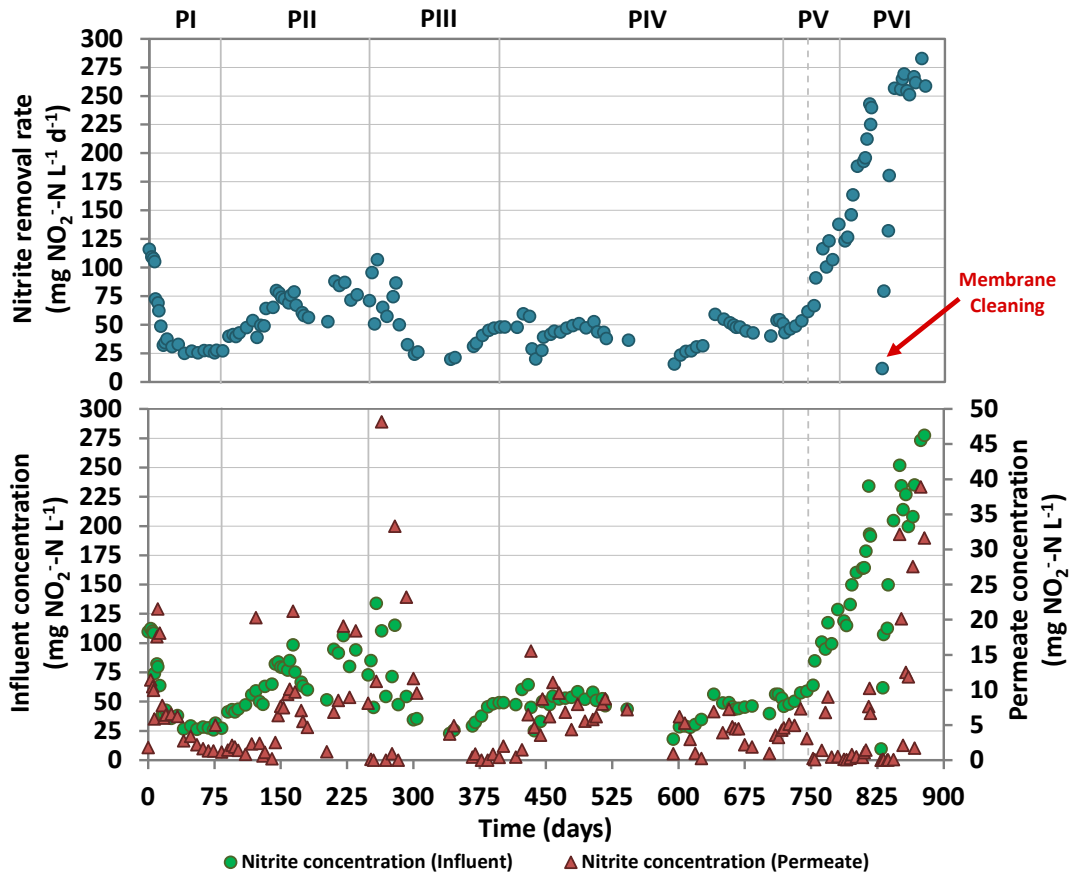

Fig. 2. Evolution of the nitrite removal rates observed in the membrane bioreactor (MBR) (*top*). Evolution of nitrite concentration in the influent and the permeate of the MBR (*bottom*). The vertical lines separate different operating periods. On day 745, the biofilm attached to reactor walls and the hollow fibers of the membrane were resuspended (vertical dashed line). On day 817, the membrane was cleaned. P represents the period.

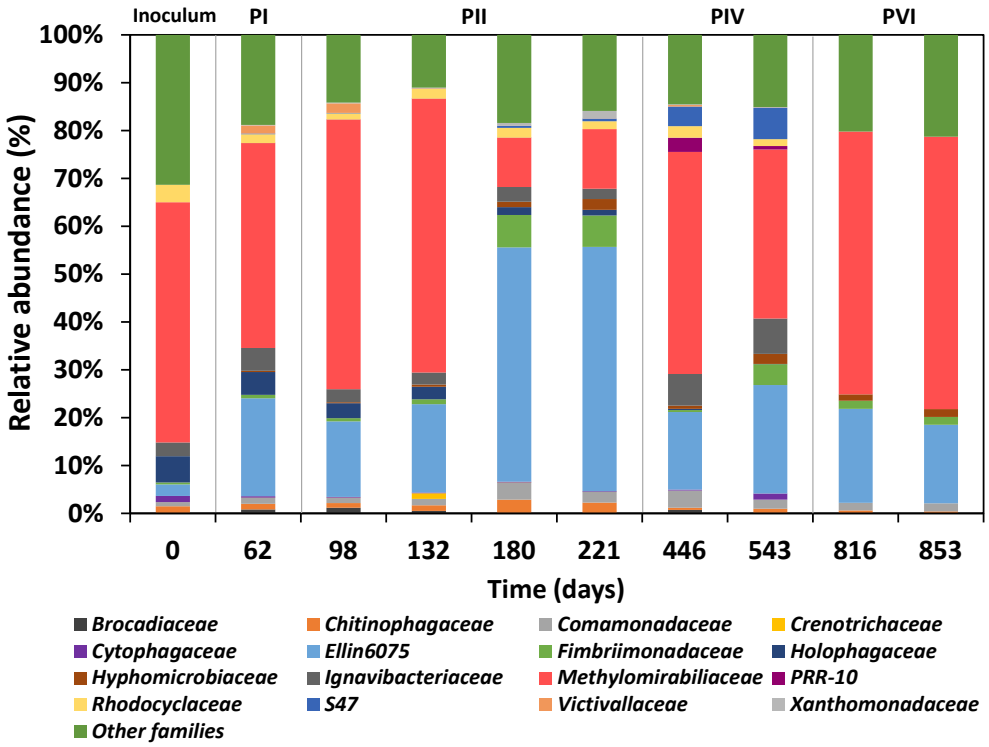

Fig. 3. Microbial diversity profiles of communities present in the suspended biomass of the membrane bioreactor system at different operational periods. The community composition expressed as the relative abundance of the most abundant microbial gender is shown for each sample. When the taxonomic classification of a genus was not possible, the best taxonomic classification for that microbial group is shown at the best taxonomic resolution achieved as follows: (c) class, (o) order, and (f) family.

might convert methane into methane oxidation products, which might be subsequently used by coexisting conventional heterotrophic denitrifiers, to reduce nitrite to dinitrogen gas [23]. Aerobic methanotrophs of the *Methylococcaceae* family were permanently found in the culture during the study but represented less than 0.07 %. During this PII, with the addition of dissolved oxygen, their abundances did not increase.

By using stoichiometric analysis (Eq.3) and considering that all the dissolved oxygen was used for AMO-D, 8.3 mg O_2 L^{-1}, a maximum nitrite removal of only 4.9 mg NO_2^--N L^{-1} d^{-1} could be justified by this process. Meanwhile, and considering that all the ammonia fed, 8 mg NH_4^+-N L^{-1} d^{-1}, the anammox process (Eq. 2), could explain up to 10.6 mg NO_2^--N L^{-1} d^{-1}. Anammox bacteria (*Brocadiaceae)* were detected during this period, but with low and decreasing relative abundances across time, from 1.16 % (day 98) to 0.04 % (day 221). AMO-D and anammox together could jointly justify nitrite removal rates of 15.5 mg NO_2^--N L^{-1} d^{-1}, however, 62 ± 16 mg NO_2^--N L^{-1} d^{-1} were achieved in PII (Number of measurements in this period, N = 30). This suggests that most of the nitrite removal might have been carried out by *N*-damo bacteria.

The family *Methylomirabiliaceae* (*N*-damo bacteria) dominated the bacterial community at the beginning of PII, representing around 57 % (Fig. 3). The dominance decreased at the end of the period, but still with values above 10 % maintained for more than 40 days. Hence, the addition of dissolved oxygen improved somehow nitrite removal, but the reason could not be elucidated. Nevertheless, the nitrite removals attained in PII are still far from PI. The positive impact of dissolved oxygen was corroborated during PIII (same experimental conditions as PI) since once the dissolved oxygen was desorbed from the feeding bags, the nitrite removals dropped.

There is still a lack of insight regarding the impact of dissolved oxygen on *N*-damo. The presence of dissolved oxygen in feed has been reported to impede the development of *N*-damo bacteria in an MBR, using an inoculum with negligible activity [15]. This indicates that oxygen should be avoided in the start-up of *N*-damo reactors. Luesken et al. [24] evaluated the short-term effect of different oxygen concentrations in a culture enriched in *N*-damo bacteria using discontinuous tests. The headspace of the bottles contained methane and oxygen (2 and 8 %) at 30 °C. In those experiments, a negative impact of oxygen in the nitrite removal was observed, with a reduction in the nitrite removal of 57 and 81 %, respectively. Kessel et al. [25] introduced a gradually increased concentration of oxygen in the influent gas (up to 5.4 %) of an SBR containing anammox and *N*-damo microorganisms. All the oxygen was consumed by the culture. Before the addition, the culture was dominated by *N*-damo bacteria, however, after 9 operating months, the amount of *N*-damo counts in the sludge decreased, and the anammox activity increased. Attempts to determine the possible activity of aerobic methane oxidation were made by these authors. The results were negative, and probably oxygen was used by ammonia oxidizers. As in the present study, the addition of oxygen did not cause any increase in aerobic methanotrophs or nitrifiers.

Liu et al. [26] studied the impact of dissolved oxygen in a biofilm growing onto the membrane of an MBfR, that couples partial nitritation, anammox, and *N*-damo processes. Oxygen was continuously provided in the liquid phase and tightly regulated, to oxidize ammonium into nitrite. Methane and carbon dioxide gas mixture was provided in the lumen of the membranes. Measured dissolved oxygen was between 0.02 and 0.1 mg O_2 L^{-1}. No negative impacts on the nitrogen removal capacity were detected. They were able to achieve high nitrogen removal efficiencies and NRRs of 98 % and 1.5 g N L^{-1} d^{-1}, observing the coexistence of *N*-damo bacteria and archaea, anammox, and aerobic ammonia oxidizers in a stratified biofilm.

In denitrifying bioreactors, nitrogen anions often are recycled in a stream containing a certain amount of oxygen dissolved in water. The results observed in the referred and the present study, showed that these microorganisms can withstand traces of oxygen in bioreactors once *N*-damo bacteria activity has been developed. This could make feasible the development of new biological wastewater treatment processes based on *N*-damo bacteria.

3.3. Addition of diluted seawater and reduction of macronutrients concentration in the feed

During PIV (days 397 and 717), the feed medium was diluted with filtered seawater at different ratios to assess if some of the ions present in seawater could be missing in the medium proposed, therefore limiting the *N*-damo bacteria activity. Two different dilution ratios (seawater: feed) were tested, 1:20 v/v (days 397–590) and 1:100 v/v (days 591–717), however, no apparent effects on the *N*-damo bacteria activity were detected. The nitrite removal rates varied between 15.8 and 59.6 mg NO_2^--N L^{-1} d^{-1}. In addition, a raise in the inorganic suspended solids concentration was detected during PIV (Table 2). The fraction of MLVSS represented 71.2 % of the mixed-liquor total suspended solids (MLTSS) during PIV, suggesting an accumulation of inorganic precipitates in the MBR, 28.8 %. It was postulated that such high a fraction of inorganics could negatively affect the denitrification potential of *N*-damo bacteria. As a result, seawater additions were stopped from day 716 until the end of the operation.

Besides the seawater additions, the high concentration of P, K, and Ca present in feed could have also contributed to the accumulation of inorganics in the MBR system. Accordingly, the concentration of $CaCl_2$ and KH_2PO_4 in the feed was reduced by 10 times from day 718 until the end of the experimentation. The same strategy has been previously conducted in our laboratories to avoid the precipitation of calcium phosphates, also in an MBR, but in that case with biomass enriched in anammox [27]. In that study, the P and Ca concentrations in feed were reduced by 40 (from 226 to 5.65 mg KH_2PO_4 L_{Feed}^{-1}) and 5 times (from 50 to 10 mg $CaCl_2{\cdot}2H_2O$ L_{Feed}^{-1}), compared to the mineral medium recommended for anammox systems [28], respectively. That reduction in the macronutrients concentration resulted in an extraordinary increase in the total nitrogen removal rates from 10 to 700 mg N L^{-1} d^{-1}.

After removing the seawater additions and reducing the macronutrients concentration in the feed, the NRRs increased from 43.2 to 66.7 mg NO_2^--N L^{-1} d^{-1} (from day 718 to 745). Moreover, the organic matter fraction in the MLTSS increased from 71 to 84 %, compared to PIV (Table 2), indicating a lower presence of inorganic suspended solids in the MBR. On that day 745 (PV), significant biofilm was detected adhered to the reactor walls. To minimize future biofilm accumulation, an operating strategy was conducted (once per week) from that day until the end of the experimentation. High flows of N_2 gas were applied for 5 min, by using a gas line submerged in the mixed liquor and placed at the bottom part of the MBR. During the process, the reactor was stopped, and positive pressure in the gas phase was guaranteed to avoid possible oxygen inlets. Once the biofilm was resuspended, a fivefold increase in the suspended biomass concentration was observed, from 0.6 to 2.14 g MLVSS L^{-1} (Fig. S3).

As a result of both strategies, the mineral medium adjustments, and the biofilm resuspension, the activity increased until achieving stable

Table 2
The organic and inorganic fractions in the mixed-liquor total suspended solids (MLTSS) at the different operating periods.

Operating Period	Organic fraction in MLTSS (%)	Inorganic fraction in MLTSS (%)
Allegue et al. [15]	88.2 ± 5.4	11.8 ± 5.4
I	84.2 ± 1.1	15.8 ± 1.1
II	80.5 ± 6.6	19.5 ± 6.6
III	79.5 ± 3.9	20.5 ± 3.9
IV	71.2 ± 4.2	28.8 ± 4.2
V	84.3 ± 0.8	15.7 ± 0.8
VI	87.8 ± 3.3	12.2 ± 3.3

NRRs of 117 ± 13 mg NO_2^--N L^{-1} d^{-1}, between days 762 and 786. These rates are similar to the maximum values previously reported by Allegue et al. [15]. By the end of PV, nitrite concentrations of only 0.5 mg NO_2^--N L^{-1} have been detected in the permeate, which could suggest a possible substrate limitation for *N*-damo bacteria.

3.4. Impact of EDTA addition on the trace elements bioavailability

On day 655 (PIV), the trace elements composition in feed and permeate were analysed by using ICP-MS. The results showed that most of the trace elements were present in the feed, however, some of them were in much lower concentrations (Fig. 4) than initially planned (Table 3). Concentrations of only 4.5 μg Fe^{2+} L^{-1} and 4.7 μg Cu^{2+} L^{-1} were measured that day in the feed, values which are 44 and 14 times lower than expected, respectively. Besides, Fe^{2+} was not even detected in the permeate of the MBR. All of this could have led to a nutrient limitation for the culture. Cu^{2+} and Fe^{2+} play a significant role in the *N*-damo bacteria metabolism since they act as indispensable elements of microbial enzymes. *N*-damo bacteria use metalloenzymes in their metabolism such as particulate methane monooxygenase (Cu-dependent), to activate methane oxidation, and cd1 nitrite reductase (Fe-dependent), to activate nitrite reduction [29,30].

To stimulate *N*-damo bacteria, several studies have focused on the trace elements composition. Kampman et al. [14] observed in an MBR enriched in *N*-damo bacteria a slight increase in the nitrite removal capacity of the system (from 13 to 16 mg NO_2^--N L^{-1} d^{-1}) after increasing the Cu^{2+} concentration from 190 to 381 μg L_{Feed}^{-1}. He et al. [30] found that increases in the concentration of both elements, Fe^{2+} (from 223 to 1,117 μg L_{Feed}^{-1}) and Cu^{2+} (from 63.5 to 635 μg L_{Feed}^{-1}), improved by three times the nitrite conversion rate of *N*-damo bacteria in long-term experiments. However, it should be considered that Cu^{2+} concentrations above 635 μg/L could inhibit *N*-damo bacteria [30]. In the same study, increases in the concentration of other elements such as Zn^{2+} (65–130 μg/L), Mo^{2+} (19–191 μg/L), Co^{2+} (14–118 μg/L), Mn^{2+} (27–110 μg/L), and Ni^{2+} (5.8–58 μg/L) did not show any impact on the activity.

The lower concentration of some elements observed in this study could be explained by the accumulation of precipitates in the acidic trace elements solution (see section 2.1) used to prepare the feed. To increase the bioavailability of the trace elements in the system a chelating agent was added only in the acidic trace elements solution from day 782 (PVI) onwards, ethylenedinitrilotetraacetic acid disodium salt (2Na-EDTA). EDTA reacts with metal ions to form stable and soluble organometallic complexes. By adding EDTA the concentration of Fe, Cu, and Zn available in the feed significantly increased (Fig. 4). The values were in the range as initially planned (Table 3). Once EDTA was added, the NRR considerably raised from 123.3 to 239.8 mg NO_2^--N L^{-1} d^{-1}, (between days 787 and 817), doubling the maximum rates accomplished by Allegue et al. [15].

Table 3
Comparison between the mineral medium composition for *N*-damo bacteria enrichments suggested by Ettwig et al. [16] and the alternative proposed in this study.

Chemical	Ettwig et al. [16]	This study	Units
KH_2PO_4	50	5	mg L_{Feed}^{-1}
$CaCl_2$	200	20	mg L_{Feed}^{-1}
$MgCl_2{\cdot}6H_2O$	–	33	mg L_{Feed}^{-1}
$MgSO_4$	97.7	10	mg L_{Feed}^{-1}
Na_2EDTA	–	1.4	mg L_{Feed}^{-1}
B^{3+}	1.2	1.2	μg L_{Feed}^{-1}
Mn^{2+}	110	110	μg L_{Feed}^{-1}
Fe^{2+}	209.5	209.5	μg L_{Feed}^{-1}
Co^{2+}	14.9	14.9	μg L_{Feed}^{-1}
Ni^{2+}	11.8	11.8	μg L_{Feed}^{-1}
Cu^{2+}	63.8	63.8	μg L_{Feed}^{-1}
Zn^{2+}	7.7	7.7	μg L_{Feed}^{-1}
Se^{+4}	9.5	9.5	μg L_{Feed}^{-1}
Mo^{6+}	22.6	22.6	μg L_{Feed}^{-1}
W^{6+}	5.6	5.6	μg L_{Feed}^{-1}

A significant membrane fouling was detected on day 817. Consequently, the reactor was open to clean the membrane. Once the membrane was retrieved from the liquor, a high accumulation of biomass was noticed attached to the membrane's fibers. The lack of membrane backwashing might have contributed to the severe membrane fouling of the hollow fiber module. The biomass was detached from the fibers manually and put back into the system. Later, the membrane was physically cleaned with water. Once the cleaning was completed the reactor operation was resumed, and to avoid any possible inhibition by substrate, the nitrite concentration in the feed was drastically reduced from 191.5 to 9.5 mg NO_2^--N L_{Feed}^{-1}. In less than 14 d, however, similar values to the ones achieved before the cleaning were reached, and a maximum NRR of 285.7 mg NO_2^--N L^{-1} d^{-1} was attained on day 874. The microbial community was dominated by the family *Methylomirabiliaceae* (*N*-damo bacteria) during this PVI, with values always above 55 % (Fig. 3). Stable NRRs were observed in the MBR in the last 35 d of operation with an average value of 262.2 ± 8.7 mg NO_2^--N L^{-1} d^{-1}. It appears that the nitrite conversion was limited at that point, proved by the nitrite accumulation of up to 38.9 mg NO_2^--N L^{-1}. This suggests that *N*-damo bacteria could operate with nitrite concentrations close to the inhibition constant observed by He et al. [20], 57.4 mg N L^{-1}.

To the best of our knowledge, the volumetric nitrite removal rates achieved in this study are the highest reported for an enrichment culture in *N*-damo bacteria. Further research is still required to evaluate if

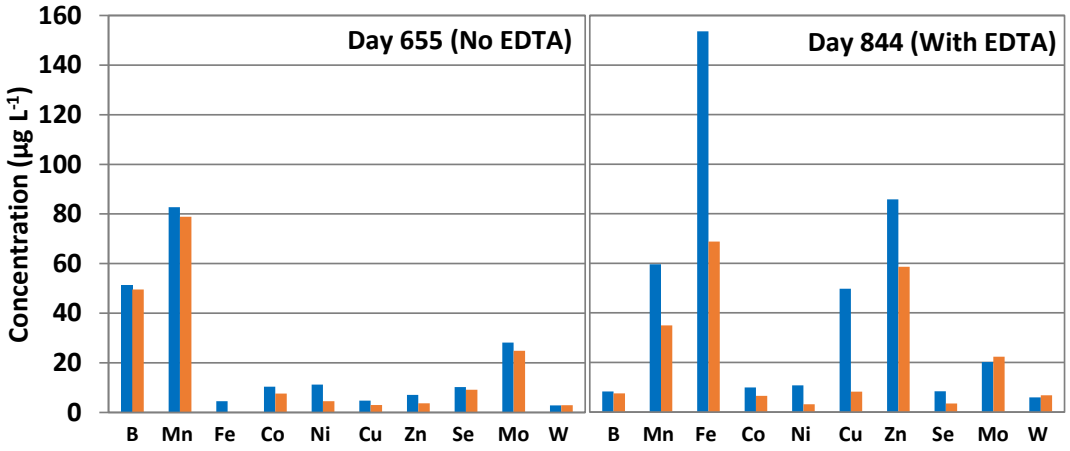

Fig. 4. The concentration of trace elements observed in samples of filtered feed and the permeate of the membrane bioreactor at different operating days: 655 (PIV), and 844 (PVI). During PVI, ethylenediamine tetraacetic acid (EDTA) was added to the feed.

higher concentrations of Cu^{2+} and Fe^{2+} in the mineral medium proposed could result in higher nitrite removal rates as observed by other authors [14,30], or otherwise, exert a negative impact on the denitrifying potential of *N*-damo bacteria. Anyway, after the modification in the mineral medium, it is demonstrated that it is possible to operate systems with biomass enriched in *N*-damo bacteria with NRR values between 100 and 285.7 mg N L^{-1} d^{-1} for more than 100 experimental days. By considering an average biomass concentration of 4.1 ± 0.3 g MLVSS L^{-1}, an apparent specific NRR of 64.1 ± 5.0 mg NO_2^--N g VSS^{-1} d^{-1} was estimated for that period. These values are much higher than those referred to in the past by Hu et al. [6], with values between 6 and 28.5 mg N g VSS^{-1} d^{-1}, and closer to those observed in one of the first enrichments reported for *N*-damo bacteria, 34–56 mg N g VSS^{-1} d^{-1} [16].

Despite the high denitrification potential observed for *N*-damo bacteria, before their practical application at industrial scale, several aspects must be addressed: i) the long start-up periods of the bioreactors must be significantly shortened; ii) the number of experimental studies to determine the biokinetic parameters of *N*-damo bacteria, required to simulate the application of the process, are currently limited; iii) the short and long-term impact of potential inhibitors of *N*-damo bacteria (e.g., oxygen, sulfide, etc.) and different experimental conditions (e.g., salinity, pH, trace elements bioavailability, etc.) must be carefully evaluated. Besides, experimental studies using pilot-scale plants have not been carried out yet and there is only a study using real wastewater [31].

4. Conclusions

This study aimed to optimize the nitrite removal capacity of a lab-scale MBR enriched in *N*-damo bacteria by using different operating strategies for 878 days. Two main factors contributed to the high and stable NRRs achieved. Firstly, compared to the mineral medium most used in other *N*-damo bacteria studies, lower concentrations of macronutrients (Ca, K and P) in feed were provided. This strategy considerably minimized the accumulation of inorganic precipitates inside the MBR. These inorganics could have a negative impact on the denitrifying activity of the system. And secondly, EDTA was added to feed to avoid the precipitation in the feed of trace elements such as Fe^{2+} and Cu^{2+}, both playing a crucial role in the metabolism of *N*-damo bacteria. The addition of EDTA significantly increased the Fe^{2+} and Cu^{2+} bioavailability. Overall, the new mineral medium proposed in this study for *N*-damo bacteria enrichments resulted in remarkable and prolonged nitrite removal rates, up to 285.7 mg NO_2^--N L^{-1} d^{-1}. *N*-damo bacteria dominated the microbial community, up to 57 %. The rates achieved are the highest reported in the literature in an *N*-damo bacteria enrichment.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2022.138289.

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