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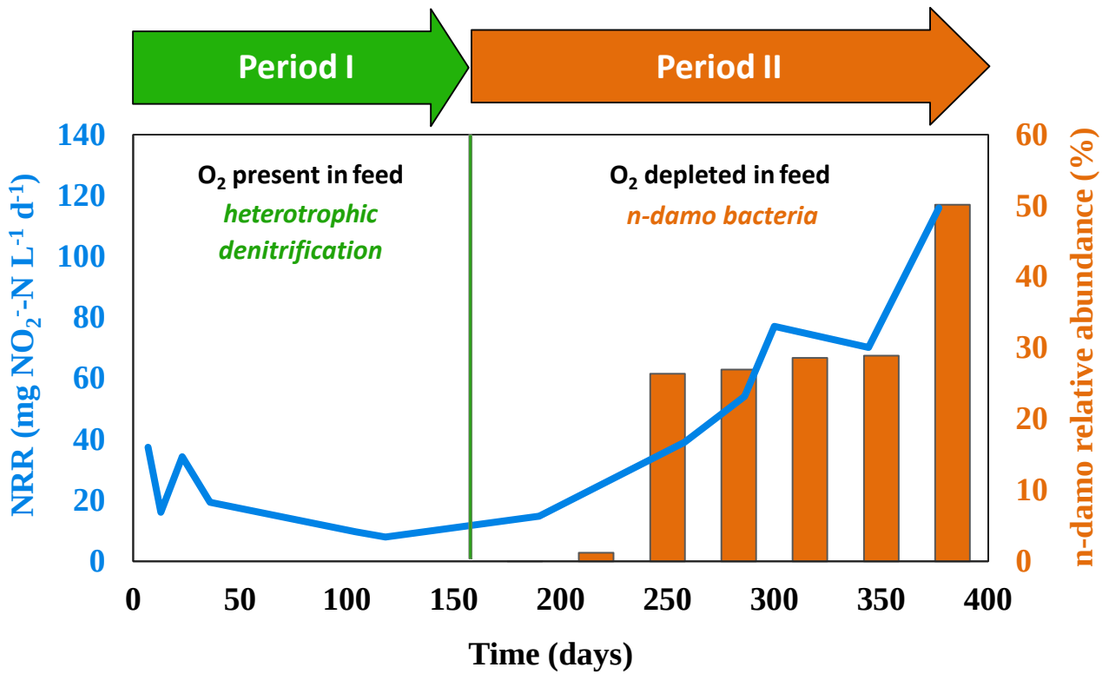
Graphical Abstract



Highlights

- The biomass enrichment on n-damo bacteria was successfully achieved
- High specific n-damo activities were attained
- Biomass accumulation was not detected during the operation
- Ammonium was added as an extra nitrogen source for n-damo bacteria growth
- Correlations between nitrite permeate concentration and N_2O production were found

Enrichment of nitrite-dependent anaerobic methane oxidizing bacteria in a membrane bioreactor

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ABSTRACT

The use of nitrite-dependent anaerobic methane oxidation (n-damo) processes could represent an innovative technology in order to minimize the environmental impact of anaerobic sewage effluents at low temperatures, since these biological processes are able to simultaneously remove nitrite and dissolved methane in anaerobic conditions. Nevertheless, n-damo bacteria are well-known by their reported low activity and slow doubling times which hinders a practical application. On this study, the enrichment on these bacteria was successfully achieved in a membrane bioreactor system at 28ºC. Despite biomass accumulation was not detected, a high apparent specific n-damo activity of 95.5 mg NO_2^--N g^{-1} MLVSS d^{-1} was achieved after 388 days of operation, being one of the highest nitrite

removal rates reported in the literature for n-damo cultures to date. Additionally a slow doubling time of 11.5 d was estimated. 16S rRNA gene amplicon sequencing analysis indicated that *Candidatus* Methylomirabilis became the most abundant bacterial organism by day 344 with a relative abundance of 50.2%. During the entire experiment ammonium was continuously added to the system as an alternative nitrogen source, to avoid biomass growth limitations. Finally, a relation between permeate nitrite concentrations and nitrous oxide production was found, which allows to optimize the process in terms of the minimization of both nitrogen species. The nitrous oxide emissions represented between 0 and 3.7% of the denitrified nitrogen.

Keywords

Methanogenic reactors; n-damo bacteria; dissolved methane; nitrous oxide; ammonium and membrane bioreactor.

Abbreviations

Anammox: anaerobic ammonium oxidation

CL: cluster

COD: chemical oxygen demand

CSTR: continuously stirred tank reactor

DAMO: denitrifying anaerobic methane oxidation

DO: dissolved oxygen

DOC: dissolved organic carbon

GHG: greenhouse gas

GWP: global warming potential

FISH: fluorescence *in situ* hybridization

HRT: hydraulic retention time

MBfR: membrane biofilm reactor

MBR: membrane bioreactor

MLTSS: mixed liquor total suspended solids

MLVSS: mixed liquor volatile suspended solids

MSGLR: magnetically stirred gas lift reactor

N-damo: nitrite-dependent anaerobic methane oxidation

NLR: nitrite loading rate

NRR: Nitrogen removal rate

OTU: operational taxonomic units

SBR: sequencing batch reactor

1. Introduction

Anaerobic sewage treatment is widely used in warm and temperate climate regions because of different advantages such as the low sludge production and the energy recovery among others, in comparison to aerobic processes. Nevertheless, a large fraction of methanized chemical oxygen demand (COD) is present dissolved in anaerobic bioreactors effluents, especially at low temperatures. Since methane is a strong greenhouse gas (GHG), with a global warming potential (GWP) 28 times higher than the CO_2 for a hundred year time horizon [1], the removal of dissolved methane from anaerobic effluents should be achieved

preventing its direct release to the atmosphere [2]. Another important challenge for these effluents is their high total nitrogen concentrations. An interesting strategy to deal with both problems is the utilization of bioprocesses, in which methane is used as inexpensive electron donor for denitrification. In this scenario two main microbiological pathways can be distinguished: aerobic and anaerobic. In the first pathway, aerobic methane oxidation is coupled to denitrification, through a consortium between aerobic methanotrophs and conventional denitrifiers [3]. In the second, bacteria affiliated with the candidate NC10 phylum, such as "*Candidatus* Methylomirabilis oxyfera*"* [4] are able to anaerobically oxidize methane by using nitrite as electron acceptor, n-damo bacteria (Eq. 1). Besides, archaea like "*Candidatus* Methanoperedens nitroreducens" [5] are also able to oxidize methane in the same conditions but reducing nitrate to nitrite, damo archaea (Eq. 2). Both anaerobic pathways are collectively called denitrifying anaerobic methane oxidation (DAMO) processes.

Nowadays, n-damo processes are too far to be implemented at full-scale plants and further investigation is needed, mainly due to their reported low activities (Table 1) and their slow doubling times of 1-2 weeks [4]. A better understanding, in terms of physiology and kinetics, would be necessary to facilitate a technological application [6].

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

$$2CH + 8NO_3^- \rightarrow 8NO_2^- + CO_2 + 4H_2O \quad (2)$$

For the last several years, different studies involving n-damo microorganisms have been carried out. Luesken et al. [7] proposed the use of an anaerobic ammonium-oxidizing bacteria (anammox) and n-damo bacteria co-culture in a sequencing batch reactor (SBR), to

simultaneously remove from wastewater nitrite, ammonium, and dissolved methane. An apparent nitrite removal of 33 mg NO_2^--N L^{-1} d^{-1} (Table 1) was achieved for n-damo bacteria. Kampman et al. [8] studied a new concept to reduce the impacts of effluents from methanogenic reactors at low temperatures with presence of a considerable amount of dissolved methane [9] and nitrogen, by using n-damo processes in a SBR at 30ºC. A nitrogen removal rate (NRR) of 37.8 mg NO_2^--N L^{-1} d^{-1} was attained, although biomass was washed out from the system throughout all the experimentation. In addition, Hu et al. [10] studied the impact of different reactor configurations in n-damo bacteria enrichments: continuously stirred tank reactor (CSTR), sequencing batch reactor (SBR), and a magnetically stirred gas lift reactor (MSGLR), achieving NRRs of 26.4, 11.4 and 76.9 mg NO_2^--N L^{-1} d^{-1}, respectively. These authors suggested that the higher nitrite removals observed in the MSGLR were the result of an improvement in the gas (CH_4)-liquid mass transfer, however, and important biomass washout was also observed with this configuration. In order to avoid the biomass washout in a n-damo enrichment culture (20ºC), Kampman et al. [11] utilized a membrane bioreactor (MBR), and, in spite of the 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}. Shi et al. [6] indicated that the low aqueous solubility of CH_4 limits n-damo activity and, in order to enhance its transfer, the use of a potential novel technology with presence of gas diffusive membranes was proposed, the membrane biofilm reactor (MBfR). The membrane surface would also promote biofilm development. These authors demonstrated for the first time the feasibility of nitrogen removal by combining damo and anammox processes, obtaining an NRR of 190 mg NO_3^--N L^{-1} d^{-1} and 60 mg NH_4^+-N L^{-1} d^{-1}. Cai et al. [12], also in a co-culture of damo

and anammox microorganisms in a MBfR, obtained a surprising nitrate and ammonium removal rate of 684 mg NO_3^--N L^{-1} d^{-1} and 268 mg NH_4^+-N L^{-1} d^{-1}, respectively.

Nitrogen is an essential nutrient for microorganisms due to its presence in macromolecules, such as, proteins and nucleic acids. In previous n-damo bacteria enrichments, nitrite was used as the only nitrogen source [8,10,11]. However, microorganisms preferentially assimilate the reduced form, ammonium, since the transformation into organic forms requires less energy than the oxidized nitrogen species [13]. In addition, authors such as Ma et al. [14] observed that low concentrations of ammonia (1-10 mg L^{-1}) stimulates the activity and the growth of *Nitrobacter winogradskyi*, a nitrite-oxidizing bacteria. On this study, despite nitrogen assimilatory pathways of n-damo bacteria has not been characterized yet, in order to avoid a possible nitrogen limitation and to guarantee a proper biomass performance, it was decided to add ammonium into the system to provide an extra nitrogen assimilation source, besides nitrite.

The main goal of this study was to promote the development of an enrichment culture of n-damo bacteria in a fully-monitored MBR, operating at 28ºC and using nitrite and methane as primary substrates. Additionally, a small amount of ammonium was fed as an alternative nitrogen source to avoid microbial growth limitations. The reactor operation was evaluated in the long-term especially focusing on the N-species removal rates (specific and volumetric) and the biomass evolution (concentration and microbial composition). Additionally, the evolution of nitrous oxide, a strong GHG, was also studied, as well as its relation to the occurrence of inhibition events.

2. Materials and methods

2.1 Reactor configuration and operation

N-damo bacteria enrichment was performed in a lab-scale MBR with 10 L of capacity, being the working volume 6.65 L. A scheme of the operation system is depicted in Figure 1. A submerged hollow-fiber ultrafiltration membrane module (Puron), with a 0.5 m^2 surface and a pore size of 0.03 μm, was used to allow a complete biomass retention in the system. The membrane was operated in cycles, with 7 minutes of permeation and 30 seconds of membrane relaxation. A pressure sensor PN2069 (IFM) provided the permeation and the relaxation transmembrane pressures. Temperature was maintained at 28ºC using a thermostatic water bath. The system was fed with the following synthetic medium: NH_4Cl, 27 mg L^{-1}; $NaHCO_3$, 100 mg L^{-1}; $NaNO_2$ ranged between 11 and 541 mg L^{-1}, depending on the biomass activity. Macronutrients and trace compounds were added accordingly to Ettwig et al. [15]. However, on day 29 the $MgSO_4$ addition was reduced around ten times, from 97.7 to 10 mg L^{-1}, to limit denitrification via sulfur-oxidizing bacteria. A 6.65 L volume of mixed suspended culture with a mixed liquor volatile suspended solids concentration (MLVSS) of 7 g L^{-1} was used as seed. The inoculum, with presence of n-damo bacteria, was taken from a denitrifying MBR system treating the effluent from a methanogenic bioreactor in which dissolved methane was used as the major electron donor for denitrification [16]. To promote n-damo bacteria enrichment, the mixing liquor was gasified with 9 L d^{-1} of a mixture containing the following composition: 95% CH_4 and 5% CO_2. On day 297 the gas flow was raised to 13 L d^{-1}. The CO_2 addition was necessary in order to provide enough buffer capacity to the system. During the operation CH_4 and CO_2 were supplied in excess into the head-space of the reactor by using a mass-flow controller

E-7000 (Bronckhorst), to avoid any buffer or electron donor limitation. In order to keep a homogeneous mixing and preventing membrane fouling, 5.5 L min^{-1} of gas were recirculated from the top to the bottom of the reactor with a mini laboratory blower N 86 KT. 18 (Laboport). The operation of the system was controlled by a programmable PLC Micro 820 (Allen-Bradley) connected to a computer.

During the first 155 days of operation the feeding bags were not deoxygenated, but from that day onwards different strategies were followed to diminish the presence of dissolved oxygen (DO): from day 155, DO was desorbed from feeding by using nitrogen gas during at least 2 minutes; from day 203, the deoxygenation time was increased up to 10 minutes, and subsequently, from day 248 the permeable-to-oxygen collapsible silicon bags were substituted by aluminum foil ones, which are totally impermeable to oxygen.

2.2 Analytical methods

Liquid and gas samples were taken from the reactor 2-3 times per week. Temperature, pH, nitrite, nitrate, ammonium, mixed liquor total suspended solids (MLTSS), MLVSS, and dissolved organic carbon (DOC), were determined according to Standard and Methods [17]. DO in feeding bags was analyzed by using a multi-parameter meter (Hach HQ40d), with a luminescent optical probe (IntelliCAL LDO101). A Mili GasCounter MGC-1 V3.3 PMMA (Ritter) was used to measure the daily added gas. An online Ultramat 23 gas analyzer (Siemens) allowed to determine the nitrous oxide production from day 104 onwards.

Culture-independent techniques were used to characterize the microbial community on 13 different days when homogeneous biomass was withdrawn from the reactor and fixed in 4% v/v paraformaldehyde for fluorescence *in situ* hybridization (FISH) or immediately frozen for DNA extractions. FISH was performed as previously described Regueiro et al. [18] with probes specific for n-damo bacteria, n-damo archaea, anammox bacteria and the domain archaeal probe. For the sequencing of the 16S rRNA gene the protocol described by Regueiro et al. [19] was followed with few modifications. In brief, after extracting the bulk DNA, the V3V4 and V2V3 regions of the 16S rRNA gene were amplified for *Bacteria* and *Archaea* domains respectively, and sequenced in an Illumina MiSeq platform. Raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTU) at 97% of sequence similarity using QIIME v1.9.1 [20]. Community diversity was analyzed to measure the compositional complexity of reactor microbiome. Richness was evaluated by the estimated the number of species (S) and the Gini-Simpson index (H_{GS}) while community evenness was measured with the Simpson evenness index (E). The community similarity was determined by Bray-Curtis dissimilarities and explored by non-metric multidimensional scaling (NMDS). All statistical analyses were performed in R [21]. Finally, a co-occurring microbial network was built for the abundant OTUs (relative abundances over 0.1%) with CoNet v.1.1.1. [22]. Further details about FISH and DNA handling and sequencing as well as data processing are available in supplementary material. The obtained sequences have been deposited in the SRA database (SRA identifier: SRP131848, Study Identifier: PRJNA420651).

3. Results and discussion

3.1 MBR performance

The MBR system was continuously operated for 388 days with an average permeate flow of 6.87 ± 0.4 L d^{-1}, which corresponds to a hydraulic retention time (HRT) value of 0.97 ± 0.05 d. Transmembrane pressure values, lower than 10 mbar, and permeability values above 200 L m^{-2} h^{-1} bar^{-1}, were observed during the whole operation, indicating a good membrane performance. The pH varied between 6.25 and 8.46 while the average value and the standard deviation were 7.37 ± 0.51. Nitrite and ammonium concentration values in influent and permeate, along with the daily feed flow were used to calculate the NRRs observed in the MBR system (Figure 2). The nitrite loading rate (NLR) was adjusted based on the observed nitrite removal capacity of the culture. The operation was distinguished in two periods according to the DO presence in the feed: Period I, in which the NRR firstly diminished and secondly remained constant; and Period II, in which the n-damo activity increased after reducing DO presence in the feeding bags. Likewise, two different sub-periods have been differentiated based on the nitrite removal dynamics: Periods I-A and I-B; Periods II-A and II-B.

During Period I (experimental days 0-154), in which DO has not been purged from feeding bags, a constant permeate flow of 7.2 ± 1 L d^{-1} was attained. This period was characterized by an important biomass decay and by relatively low NRRs that ranged from 6.2 to 37.48 mg NO_2^--N $L^{-1}d^{-1}$.

Throughout the first 29 days of Period I-A (operating days 0-87) an average NRR of 28.8 ± 8.4 mg NO_2^--N L^{-1} d^{-1} was achieved, however, this value gradually decreased up to 6.2 mg NO_2^--N L^{-1} d^{-1} on day 87. During Period I-A the MLVSS concentration rapidly diminished

(Figure 3) from 7 g MLVSS L^{-1} (day 0) to only 3.1 g MLVSS L^{-1} (day 87) and the DOC average value in permeate, 12.4 ± 3.4 mg L^{-1}, was higher than that observed in influent, 4 ± 2.7 mg L^{-1}. Thus, DOC and MLVSS evolution indicated that most of the biomass was lost as result of decay processes. From day 62 onwards, DOC values in feeding and permeate were practically similar. The presence of decay products used by conventional heterotrophic denitrifiers to denitrify, and not anaerobic methane oxidizers, could be responsible for at least 93% of the observed nitrite removal. This value was calculated assuming a COD/MLVSS ratio of 1.42 g g^{-1}, and a COD/NO_2^--N ratio of 1.71 g g^{-1} for denitrification using nitrite as electron acceptor [23].

Period I-B (operating days 88-154) was characterized by low nitrite and negligible ammonium removal rates, and by further reduction in biomass concentration, from 3.1 to 2 g MLVSS L^{-1}. In this period, the NRRs have been stabilized, obtaining an average value of only 8.4 ± 0.9 mg NO_2^--N L^{-1} d^{-1}. The lower nitrite removals obtained during this sub-period could be explained by the lower availability of biomass decay products for endogenous denitrification. Using COD balances, it was estimated that all the nitrite removal observed in this period could be explained by conventional denitrification processes.

During Period II (days 155-388), in which a constant permeate flow of 6.8 ± 0.3 L d^{-1} was maintained, different strategies were followed to diminish the presence of oxygen in feeding bags. Simultaneously, nitrite consumption started to rapidly increase from only 2.6 mg NO_2^--N L^{-1} d^{-1} (day 174) until a maximum of 116 mg NO_2^--N L^{-1} d^{-1} (day 388), which represents the highest NRR value achieved during the experimentation. Throughout this period the MLVSS concentration slowly diminished from 2 to 1.1 g L^{-1}, and considering the

observed loss of biomass concentration, only 8.3% of the denitrified nitrite could be explained by conventional denitrification processes using decay products.

Period II-A (days 155-239) was characterized by a significant increase in the NRRs in comparison to Period I-B, obtaining a stable value of 17.2 ± 4.5 NO_2^--N L^{-1} d^{-1}, 20 days after diminishing the DO presence in feeding bags. Between days 191-239, nitrite was not detected in permeate (Figure S1), probably limiting the maximum NRR that could be attained. In contrast, ammonium consumption was not detected yet.

In Period II-B (days 240-388), biomass concentration slightly dropped from 1.9 to 1.1 g MLVSS L^{-1}. Between operating days 240-302, the NRRs began to considerably increase from 20.1 to 75.9 mg NO_2^--N L^{-1} d^{-1}, accordingly, pH value raised from 7.65 to 8.45. On day 307 a strong inhibition event in the MBR system took place. A nitrite concentration of 51.9 mg N L^{-1} was observed in the effluent, while the grab permeate sample presented a pH value of 8.3 that day. Considering the nitrite concentration in both, permeate and influent, a NRR of only 10.2 mg NO_2^--N L^{-1} d^{-1} was determined. As soon as the high permeate nitrite concentration had been detected, the reactor feeding was stopped for 45 hours with the purpose of studying the nitrite consumption evolution over time. During those hours the nitrite concentration in the mixed liquor was slowly reduced from 51.9 to 23.9 mg NO_2^--N L^{-1}, showing a residual nitrite removal rate of 14.9 mg NO_2^--N L^{-1} d^{-1}, which indicates that the system was partly inhibited.

The denitrification of nitrite by using methane generated hydroxyl ions (Eq. 1) that were counteracted especially by the presence of CO_2 in the used gas mixture. A change in the gas composition on day 302 may possibly explain the sharp drop in nitrite removal. By the first

and only time, due to a delay in the reception of the gas cylinder replacement, the gas cylinder (95% CH_4 and 5% CO_2) was replaced by one containing pure methane. Despite 200 mg L^{-1} of sodium bicarbonate ($NaHCO_3$) were continuously present in influent, the buffer capacity was not high enough to avoid a sharp drop in denitrifying activity. Due to the lack of an online pH measurement, it was not possible to determine the pH inside the reactor at the inhibition moment. To solve this unknown, a chemical equilibrium model, using Visual Minteq allowed to calculate the pH, considering that denitrification was not affected during the first hours after the cylinder replacement. A value of 11 was predicted, and probably the process was partly inhibited at lower pH value. He et al. [24] studied the short-terms effects of pH (range 6-9) in n-damo bacteria, concluding that its activity would be considerably inhibited at a pH of 9. Thus, if the predicted pH value of 11 was present in the medium, n-damo bacteria could have seen their activity inhibited with the subsequent nitrite accumulation in the MBR (52 mg NO_2^--N L^{-1}), that further inhibited the microorganisms. He et al. [25] estimated a nitrite inhibition constant for n-damo bacteria of 57.4 ± 7 mg NO_2^--N L^{-1}.

Considering that high nitrite concentrations could inhibit n-damo bacteria, it was decided to wash out all nitrite from the system by feeding the reactor with the same medium composition, but in absence of nitrite. Once the nitrite was completely removed from the mixed liquor and the gas cylinder (100% CH_4) was replaced by the mixture (95% CH_4; 5% CO_2), a very low nitrite loading rate of 2.6 mg NO_2^--N L^{-1} d^{-1} was applied. In a short period of time the biomass activity was recovered, and therefore, the nitrite loading rate was gradually increased by adjusting the nitrite addition. By day 388 (81 days after the inhibition event) the maximum nitrite removal rate of 116 mg NO_2^--N L^{-1} d^{-1} was achieved.

Doubling time of n-damo microorganisms (t_d) (Eq.3) was calculated considering the increase on NRR observed from day 279 to 302 (Eq. 4). The permeate nitrite concentration observed between these days ranged from 0.83 to 5.24 mg N L^{-1}, values higher than the nitrite affinity constant for n-damo bacteria of 0.28 mg NO_2^--N L^{-1} [25], apparently, not limiting the n-damo growth rate. A t_d of 11.5 d has been estimated, by linear correlation of the evolution of the logarithm of NRR with time, accordingly with Eq. 4.

$$t = \frac{\ln [r_s/r_{s0}]}{\mu_m} \quad (3)$$

$$r_s = \frac{\mu_m XS}{Y \cdot (K_s + S)} \quad (4)$$

Where r_s is the observed nitrite removal rate (mg L^{-1} d^{-1}); r_{so} is the NRR at operating day 279; t is time, considering as day 0 the operating day 279. μ_m is the maximum specific growth rate; X represents the biomass concentration; S the substrate concentration (nitrite); and K_s is the nitrite affinity constant.

3.2 Microbial community analysis

FISH analysis of the MBR biomass indicated an enrichment on n-damo bacteria, first detected on day 190, until the end of the operational time, when n-damo bacteria comprised a significant fraction of the cells (Figure 4 a-d). In addition, these data showed a scarce presence of anammox bacteria towards the end of the operational time and an overall low abundance of archaeal cells, which were sporadically observed. To further study the composition and temporal changes of the microbial community of the MBR, partial 16S

rRNA gene sequences were analyzed in the inoculum and at 12 different times during the reactor operation. After quality filtering, a total of 670,887 bacterial and 593,423 archaeal high quality sequences were considered for further analysis (Table S1). Sequences were grouped into Operational Taxonomic Units (OTU), which were defined as predominant when their relative abundances exceeded 1%, and as abundant when the values were larger than 0.1%. Microbial community was taxonomically very diverse. Bacterial OTUs were distributed in 50 different *phyla*; among which *Chlorobi*, *Bacteroidetes*, *Proteobacteria*, NC10 and *Acidobacteria* were the most abundant groups (Figure 4e); with species scattered over more than 300 different families of which *Saprospiraceae*, an unknown *Chlorobi* SJA-28 family and *Methylomirabiliaceae* were the most abundant ones (Figure 4f). *Archaea* organisms were mainly allocated into ten predominant methanogenic genera within the *Euryarchaeota* phylum that, on average, represented the 99.2% of the archaeal community (Figure S2). Diversity estimations showed a microbial diversity loss during the reactor operation when the number of observed archaeal and bacterial species gradually decreased from period I-B to II-B while community uniformity dropped only at the end of Period II-B (Figure S3).

The MBR microbial community was dynamic and underwent changes through time that closely followed the different operational periods (Figure S4). To better determine the temporal trends of microorganisms across time, abundant OTUs co-occurrence network was calculated and clusterized to find groups of organisms with similar temporal patterns of abundance. Using this approach, nine groups co-occurring microorganisms, called clusters (CL), were found (Figure S5), five of which contained more than three OTUs and that were further analyzed. Clusters had unique temporal trends and taxonomic compositions (Figure

5). The MBR microbial community was dominated by different clusters during each operational period. During period I-A, *Bacteria* community was mainly comprised by organisms of CL1, 2 and 4 that together represented the 65.0-67.0% of *Bacteria* domain, specifically *Saprospiraceae* family and *Chlorobi* SJA-28 class that were the 30.4 and 24.8% of the *Bacteria* in the inoculum respectively (Figure 5a, Table S2). CL1 and 4 were closely related (Figure S5) and shared some taxonomic similarities. Both clusters were dominated by different strains of *Saprospiraceae* family and *Methylocaldum* genera, although CL4 was more diverse than CL1 and included strains of *Anaerolinea*, *Chloracidobacteria*, *Fimbriimonas*, *Comamonadaceae* and *Methylotenera mobilis* among others (Figure 5b, Table S2). In contrast, CL2 was mostly composed by *Chlorobi* SJA-28. In period I-B, CL2 and 4 abundances were maintained or slightly increased while values of CL1 dropped and CL5 increased. On average, CL2, 4 and 5 represented the 64.0% of *Bacteria* during this period. As in the case of CL2, CL5 diversity was low as it was mostly composed of *Chitinophagaceae* strains. As a result, *Saprospiraceae* family members became displaced by the new microorganisms during Period I-B.

Period II-A was characterized by a severe drop of CL4 relative abundances and an increment in CL 2 and 5 values. Because of it, bacterial community was highly dominated by *Chlorobi* SJA-28 and *Chitinophagaceae* strains by day 190. But it was during Period II-B when *Bacteria* community underwent its largest shift. This period was characterized by the severe rise of CL3 OTUs and the drop of other clusters, setting aside microorganisms highly abundant during Period I, such as *Saprospiraceae* or *Methylocaldum* sp. CL3 was dominated by *C*. Methylomirabilis, a n-damo bacteria which relative abundances were negligible during Period I and low during Period II-A (1%). Despite of that, *C*.

Methylomirabilis ranged between 26.4 and 50.2% of *Bacteria* in period II-B, becoming the most abundant bacterial organism by day 344. In particular, *C.* Methylomirabilis and *Chlorobi* SJA-28 (CL2) showed opposite behaviors (Figure 5) during period II. Other organisms, including *Holophagaceae*, *Rhodocyclaceae*, *Ignaviabcteriaceae* and *Deltaproteobacteria* NB1, raised at the same time than *C*. Methylomirabilis (Figure 5b).

Nitrite consuming bacteria other than *C*. Methylomirabilis were also present in the reactor, although never in large abundances. Groups such as *Commanomadaceae* and *Hyphomicrobium* that are denitrifying organisms; or *Nitrospira* sp a nitrite oxidizer, presented their larger abundances during Period I (Table S2). Also, different strains of *C. Brocadia* peaked at Period I-B or towards the end of Period II-B. *Archaea* community underwent temporal changes that mainly consisted in changes of the dominant type of methanogen. All large clusters but CL2 contained archaeal OTUs (Table S2). In Period I-A the most predominant methanogens were C. *Methanoregula* and *Methanosaeta*, although the later was displaced during Period I-B by C. *Methanomethylovorans*. In Period II archaeal community was dominated by *Methanobacterium*.

3.3 Influence of environmental and operational conditions on biomass development

Due to the slow doubling times of n-damo bacteria, an MBR configuration was necessary to ensure complete biomass retention. The biomass was present as suspended flocs in the mixed liquor, in contrast, biofilms were not detected adhered to the glass reactor walls. However, biomass accumulation was not detected during the whole operation. In fact, MLVSS concentration continuously decreased in the MBR. This indicated that decay

processes had a higher impact in MLVSS evolution than biomass growth under our experimental conditions. The biomass decay was especially high in Period I. Nevertheless, in Period II this negative trend was considerably reduced, and at the end of it, biomass concentration was apparently stabilized. The inoculum was taken from a two stages anoxic-aerobic MBR, post-treating the effluent from a first UASB system, in which the biomass was subjected to alternating anoxic and aerobic conditions [16]. Due to the new environmental conditions that the inoculum faced, where anoxic conditions were continuously maintained, the microorganisms that were not able to adapt themselves, released biomass decay products to the system that could be used to denitrify nitrite by endogenous denitrification mechanisms. Indeed, according to COD balances, most of the nitrite removal in Period I could be explained by conventional heterotrophic denitrifiers, using decay products. In Period II-A, higher nitrite removals could have been presumably achieved, however, all nitrite was consumed during most of the period. Among the predominant *Bacteria* observed during Period I, only few groups, mainly *Comamonadaceae* and *Hyphomicrobiaceae* families, contain denitrifying heterotrophs [26,27], but those groups never dominated the MBR community. *Comamonadaceae* family has already been reported in n-damo enrichment cultures and reactors, although in low abundances [28]. Likewise, both families are not highly abundant in the MBR and probably these organisms transformed only a small part of the nitrogen during Period I. Based on 16S rRNA analysis, microbial community of this period was dominated by *Chlorobi* SJA-28 and *Saprospiraceae* taxa while the presence of n-damo bacteria was negligible. *Chlorobi* SJA-28 is a novel uncharacterized group of microorganisms very distant form any described species, hindering the description of its role in the community. Several studies have reported sequences related to *Chlorobi phylum* in nitrate or nitrite reduction microbial

consortia [29,30], including the dominance of *Chlorobi* SJA-28 of an anaerobic nitrite-reducing benzene degrading reactor amended with H_2 [31]. Also, *Chlorobi*-like sequences have been reported to comprise small percentages of the community in N-reducing methane-oxidizing reactors or microbial consortiums [32,33]. In a recent study *Chlorobi* SJA-28 has been proposed as a fermentative organism, facultative H_2-oxidizing autrotroph or both [34]. In this study, *Chlorobi* SJA-28 was enriched when all analysis indicated that the heterotrophic denitrification was the dominant process in the MBR. What is more, *Chlorobi* SJA-28 and *C*. Methylomirabilis had completely opposite temporal behaviors, probably indicating a competition relationship between both organisms. Based on that, we hypothesize that *Chlorob*i SJA-28 metabolism also includes some denitrifying capabilities.

Even though oxygen was continuously degasified from the reactor using a methane-carbon dioxide mixture, its presence in feeding bags during the whole Period I could have promoted some aerobic activity. On this ground, the role of *Saprospiraceae* family is more clear as this taxa is a well-known group of aerobic microorganisms frequently found in activated sludge that is involved in the decay of complex organic matter that is frequently found in activated sludge [35]. This lineage was likely contributing to the biomass endogenous decay at the expenses of the oxygen content inside the reactor. Oxygen also allowed the presence of aerobic methanotrophs and methylotrophs (i.e. *Methylocaldum* or *Methylotenera*) in the MBR community during this period.

As previously mentioned, n-damo bacteria presence was negligible during the first period of operation, probably due to the presence of DO in the feed and the competition. There are few studies in which the impact of DO on n-damo activity was determined. Luesken et al. [36] observed that a biomass highly-enriched in *C*. Methylomirabilis oxyfera was sensitive

to oxygen presence. The addition of different pure O_2 concentrations in the headspace of two test bottles, 2 and 8% at 30ºC, led to a reduction in nitrite removals of 57 and 81%, respectively. According to Henry´s Law oxygen concentrations of 0.74 and 2.97 mg O_2 L^{-1}, could be respectively expected dissolved in both bottles. However, the effect of lower additions has not been studied yet and further research would be necessary. Nevertheless, due to the severe inhibitions observed by Luesken et al. [36] it has been hypothesized that DO concentration present in feeding bags could have been high enough to inhibit the n-damo proliferation. This finding makes the accumulation of anaerobic methane oxidizers difficult for technological purposes, as DO is usually present in raw wastewaters.

This scenario changed during Period II. As soon as DO was purged from feeding bags, nitrite removal rate started to gradually increase. In parallel, the abundances of n-damo bacteria increased achieving the enrichment at the end of the operational period when *C*. Methylomirabilis became the most abundant organism in the MBR as determined by both FISH and 16S rRNA gene amplicon sequencing. The maximum value of NRR achieved during the operation, 116 mg NO_2^--N L^{-1} d^{-1} (day 388), coincided with the highest presence of *C*. Methylomirabilis in the mixed liquor. This value represents one of the highest nitrite removal rates reported in the literature for n-damo cultures to date (Table 1). Nevertheless, as will be further explained, it is believed that not all nitrite consumption was attributed to n-damo bacteria. Besides *C*. Methylomirabilis, a diverse cohort of bacteria became promoted during Period II, which mainly included lineages of anaerobic heterotrophic bacteria such as *Holophaga* [37], and methanogenic *Archaea* that are likely feeding on the products of decay of the microorganisms replaced by n-damo bacteria.

3.4 Maximum ammonium and nitrogen removal rates

During the entire experiment, ammonium has been continuously added to the system for biomass assimilation purposes, except in the last three days of operation. Ammonium concentration was limited to 8 mg NH_4^+-N L^{-1} to avoid the proliferation of anammox bacteria, microorganisms that are able to oxidize ammonium in absence of oxygen by using nitrite as electron acceptor. Anammox are known to outcompete n-damo bacteria under an excess of ammonium due to their higher nitrite affinity constant [38]. On day 286, the first time an important ammonium consumption was achieved, C. *Brocadia* was detected although at a residual relative abundance of 0.048% as determined by 16S rRNA gene amplicon sequencing analysis. Despite this low value, a significant ammonium removal rate of 8.9 mg NH_4^+-N L^{-1} d^{-1} was achieved that day. Between operating days 240-302, an average removal rate of 9 ± 0.7 mg NH_4^+-N L^{-1} d^{-1} was observed, nevertheless, at the inhibition event (day 307) not only the nitrite consumption dropped, but also the ammonium removal, achieving a low NRR value of 1.25 mg NH_4^+-N L^{-1} d^{-1}. However, as with the nitrite case, the ammonium removal rate rapidly increased, and values obtained prior to the inhibition event were accomplished on day 328. From that day onwards, steady removal rates of 8.7 ± 0.9 mg NH_4^+-N L^{-1} d^{-1} were achieved. During the whole operation ammonium was continuously detected in permeate (Figure S6) but in the last two samples (days 385 and 388), in which its concentration dropped to 0 mg N L^{-1}.

During the experimentation period, anammox presence was detected in the microbial community when ammonium was consumed. This fact could possibly indicate that anammox bacteria were responsible for most of the ammonium consumption in the MBR system. The maximum relative abundance for anammox of 0.6% was observed by day 377.

On day 388, when the highest NRR value of the study was achieved, 116 mg NO_2^--N L^{-1} d^{-1}, if it is considered that all ammonium was consumed by anammox bacteria, 9.7 NH_4^+-N L^{-1} d^{-1} , and considering a nitrite/ammonium molar ratio of 1.32:1 for these microorganisms, a maximum apparent NRR of 103.2 and 12.8 mg NO_2^--N L^{-1} d^{-1} could be expected for n-damo bacteria and anammox, respectively. The resulting apparent specific activity for n-damo bacteria, 95.5 mg NO_2^--N g^{-1} MLVSS d^{-1}, represents a promising value if it takes into account that in conventional activated sludge systems typical denitrification values of 250 mg N $gVSS^{-1}$ d^{-1} are observed [23]. As a result, if a successful biomass accumulation was achieved, a practical application for n-damo processes would be possible. Nevertheless, a slow doubling time of 11.5 d has been estimated, accordingly to the value of 1-2 weeks referred by Ettwig et al. [4].

3.5 Role of nitrite in nitrous oxide generation

From day 104 onwards, nitrous oxide production was continuously measured in the gas phase using an online gas analyzer (Ultramat 23). Even considering that n-damo bacteria do not produce N_2O [4], this gas was detected during most of the experimentation. Curiously, the appearance of nitrous oxide was associated to periods in which nitrite ion accumulated in the system. Between days 104-177, in which permeate nitrite concentrations ranged from 8.2 to 19.8 mg NO_2^--N L^{-1}, and in presence of residual denitrifying activity, the nitrous oxide production ranged from 0.13 to 1.82 mg N_2O-N d^{-1}. These measurements have allowed to calculate the percentage of nitrogen denitrified as N_2O-N of the total nitrogen removed in the mixed liquor, which varied from 0.2 to 3.7%. Furthermore, in order to find

a possible relationship, nitrous oxide concentration in the off-gas was correlated with nitrite in the bioreactor permeate, estimating a low coefficient of determination (R^2) value of 0.30 (Figure 6a). Prior to day 104 correlations were not provided since the gas analyzer had not been installed yet. Between days 178-388 (Period II), in which nitrite removal gradually increased, the permeate nitrite concentration ranged from 0 to 5.2 mg NO_2^--N L^{-1} (excluding the inhibition day) and the N_2O production varied from 0 to 6.3 mg N_2O-N d^{-1}. The percentage of nitrogen denitrified as N_2O-N of the total nitrogen removed in the mixed liquor varied from 0 to 2.4%. Correlations were studied prior (days 185-302; Figure 6b) and after (days 315 to 388; Figure 6c) the inhibition event. In both periods, R^2 values of 0.82 and 0.70 were obtained, respectively, and it is important to highlight that those days in which nitrite was not detected in permeate, nitrous oxide was not produced.

During the inhibition event, in spite of the elevated nitrite accumulation in the MBR system (52 mg NO_2^--N L^{-1}), a nitrous oxide production of solely 0.3 mg N_2O-N d^{-1} was observed. According to the equation showed in Figure 6b, a nitrous oxide production value of 23.8 mg N_2O-N d^{-1} may be expected, value which is far much higher than the observed. Furthermore, once the reactor feeding was stopped for a period of 45 hours, after the inhibition event, nitrous oxide emissions were not detected. This indicated that nitrous oxide production has been also affected.

N_2O emissions could be produced by either, chemical or biological nitrogen conversions in nitrogen removal bioreactors [39]. It is generally accepted that neither, n-damo bacteria nor anammox produce nitrous oxide [4,40]. Indeed, in this study N_2O emissions have been also observed in periods in which either, n-damo or anammox have not been detected by

molecular tools (Period I-B). Thus, other microorganisms that might coexist with n-damo bacteria are likely responsible for these emissions.

In summary, the continuous N_2O measurements provided by the gas analyzer have been used as a useful tool to instantly estimate the nitrite concentration in the MBR system, preventing from a possible biomass inhibition by nitrite accumulation. N_2O is a strong GHG with a GWP 265 times higher than CO_2 for a hundred year time horizon [1]. For that reason, it would be of vital importance to control the nitrite concentration in n-damo reactors to reduce GHG emissions.

4. Conclusions

This study provides a significant progress in the development of new biological processes to carry out the simultaneous removal of nitrogen and dissolved methane, a very common challenge derived from the increasing use of anaerobic reactors for sewage treatment. The use of a fully-monitored MBR reactor treating nitrite and dissolved methane during more than a year led to the development of a specific biomass highly enriched in n-damo bacteria (50.2%). A maximum specific activity up to 95.5 mg NO_2^--N g^{-1} MLVSS d^{-1} was achieved representing, to the best of our knowledge, one of the highest nitrite removal rates reported in the literature for n-damo cultures. In addition, a relation between permeate nitrite concentrations and nitrous oxide production was found, which could be used for both, the control of the process in terms of nitrite removal optimization and also to minimize the potential emissions of this strong GHG.

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Table

Table 1. Overview of the nitrogen removal rates reported in literature for n-damo bacteria.

References	Reactor configuration	Temperature (°C)	HRT (d)	Nitrogen Removal Rate (mg N L^{-1} d^{-1})
[41]	SBR	25	5	Nitrite: 15.3
[15]	SBR	30	13-30	Nitrite: 29.3
[7]	SBR	30	50-15	TN: 110
[8]	SFBRs	30	1.3-1.5	Nitrite: 33.5-37.8
[6]	MBfR	22	3	Nitrate: 190 Ammonium: 60 TN: 250
[42]	SBR	35	-	Nitrate: 67.7 Ammonium: 57 TN: 124.7
[11]	MBR	30	1.3	Nitrite: 36
[10]	CSTR SBR MSGLR	30	2	Nitrite: 26.4 Nitrite: 11.4 Nitrite: 76.9
[12]	MBfR	22	3-1.5	Ammonium: 354 Nitrate: 684 TN: 1038
[43]	SBR	35	29	Nitrite: 46
[32]	MBfR	35±2	4	Nitrate: 78.3 Ammonium: 26
This study	MBR	28	1	Nitrite: 116 Ammonium: 9.7 TN: 125.7

Figure captions

Figure 1. Schematic diagram of the MBR.

Figure 2. Volumetric nitrite and ammonium consumption rates in the MBR.

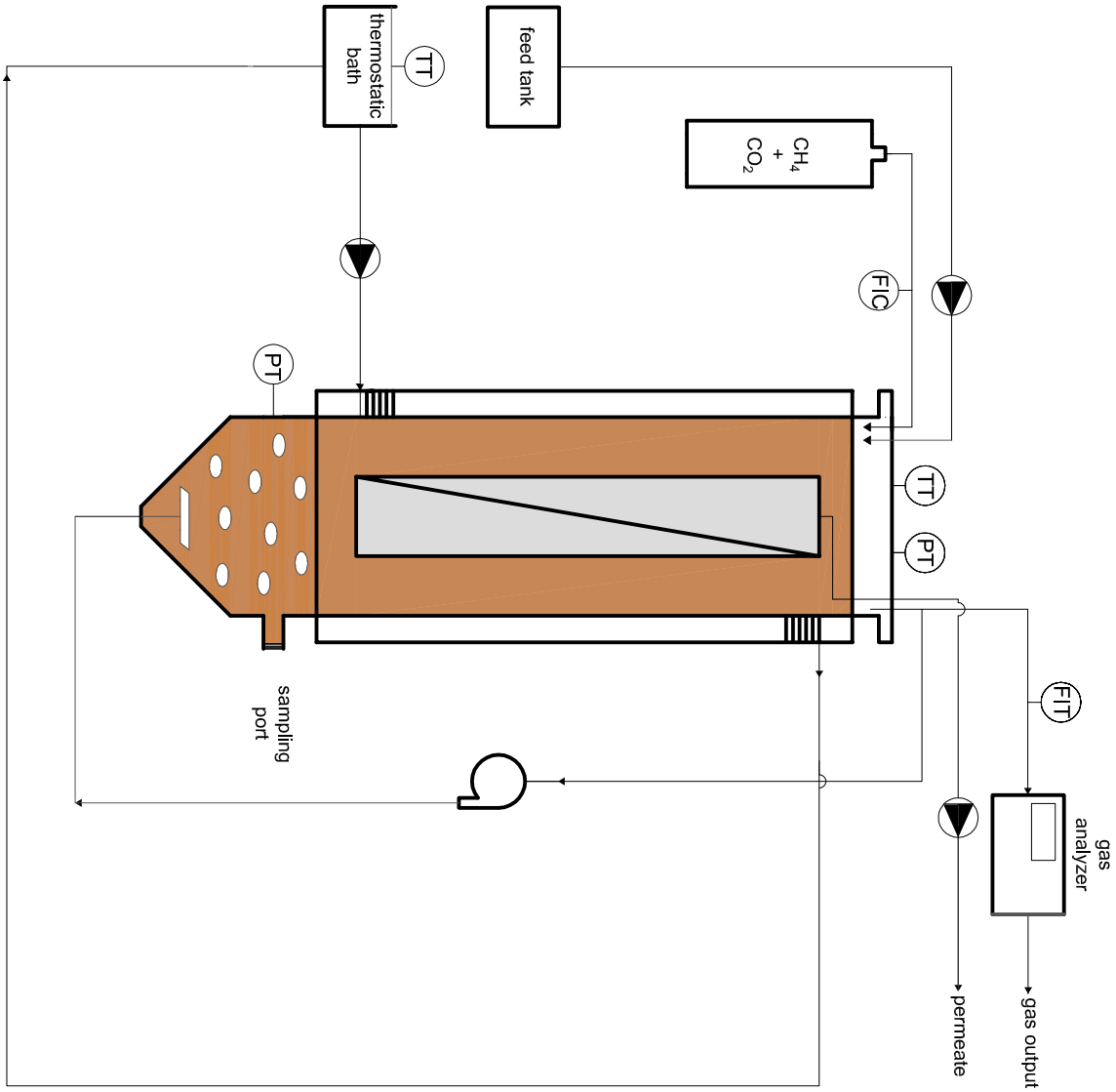
Figure 3. MLTSS and MLVSS concentration evolution in the MBR.

Figure 4. Main *Bacteria* taxa present in MBR. Presence of *C.* Methylomirabilis detected by FISH with probes for n-damo (pink) after a) 0, b) 190, c) 258 and d) 330 days of operation. All cells were stained with DAPI (blue). Scale bars indicate 10 µm. e) *Bacteria* phyla with relative abundances over 1% and f) *Bacteria* families with relative abundances over 2%. Operational periods are separated by vertical black lines. Minor category includes 37 *phyla* and 334 families respectively. _unk suffix indicates unknown.

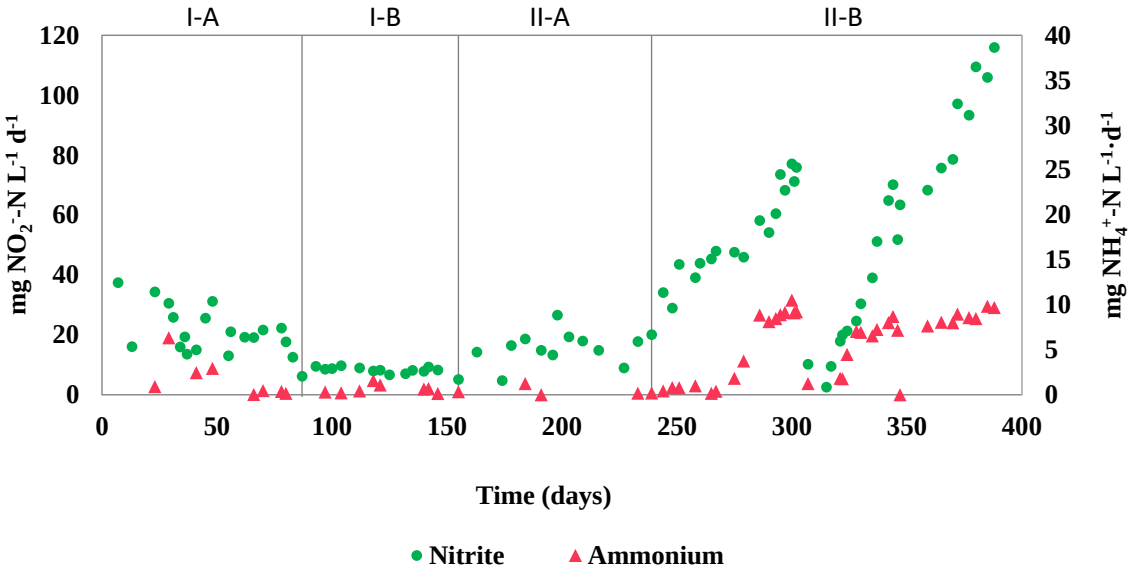
Figure 5. Temporal trends of co-occurring *Bacteria*. a) Accumulated relative abundance of all *Bacteria* OTUs within each of the 5 largest clusters of co-ocurring organisms. b) Heatmap showing the temporal changes of the most abundant OTUs from the 5 largest clusters grouped by taxonomic assigments, which detail is indicated as follows: p: phylum, o: order, c: class, f: family; g: genera. Operational periods are indicated on top of the graphs. CL indicates the cluster number.

Figure 6. Influence of permeate nitrite concentration on nitrous oxide production (measured by the online gas analyzer) in the MBR system during three different periods.

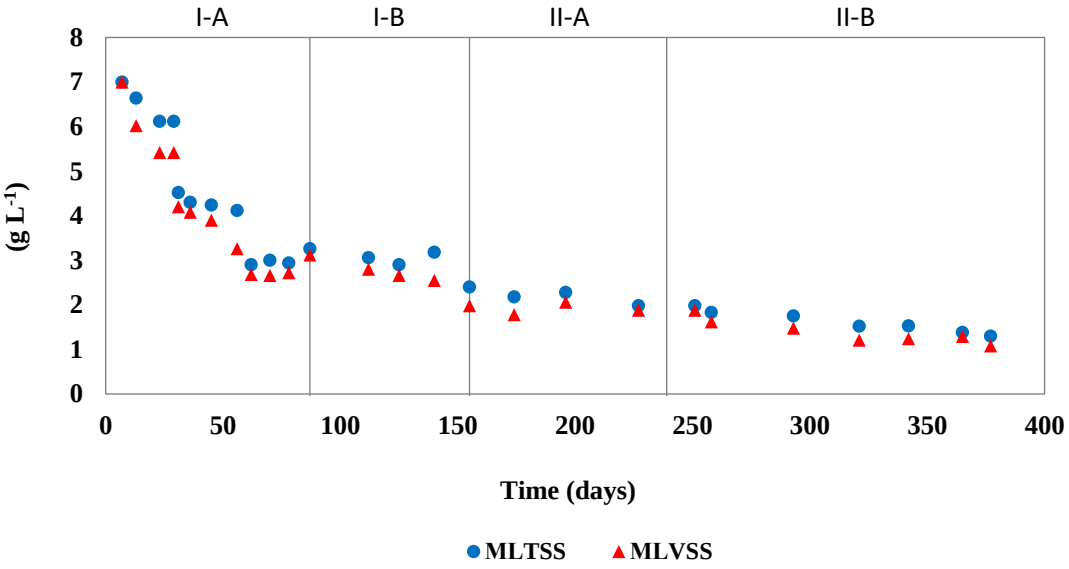
Figure

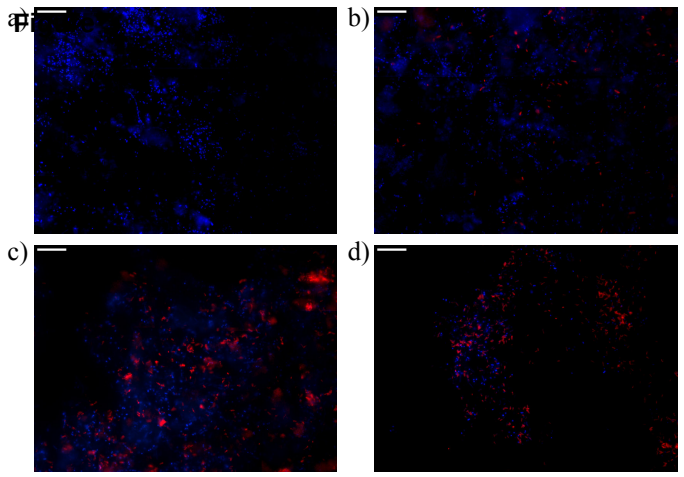

thermostatic bath
TT
feed tank
CH_4 + CO_2
FIC
PT
TT
PT
sampling port
FIT
gas analyzer
permeate
gas output

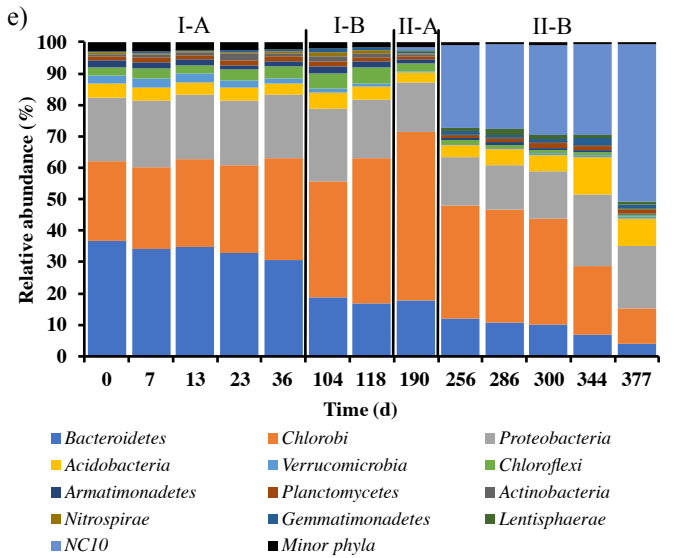
Figure

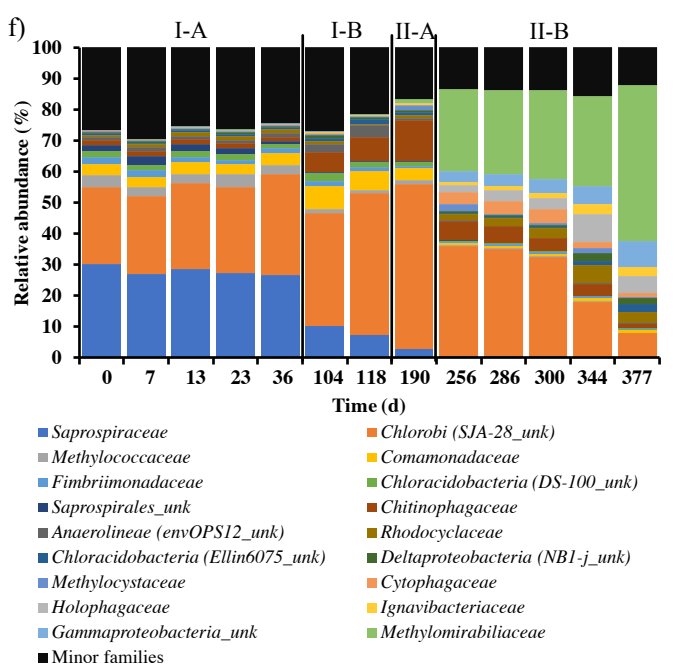

I-A
I-B
II-A
II-B
120
100
80
60
40
20
0
mg NO_2^--N L^{-1} d^{-1}
40
35
30
25
20
15
10
5
0
mg NH_4^+-N L^{-1}.d^{-1}
0
50
100
150
200
250
300
350
400
Time (days)
Nitrite
Ammonium

Figure

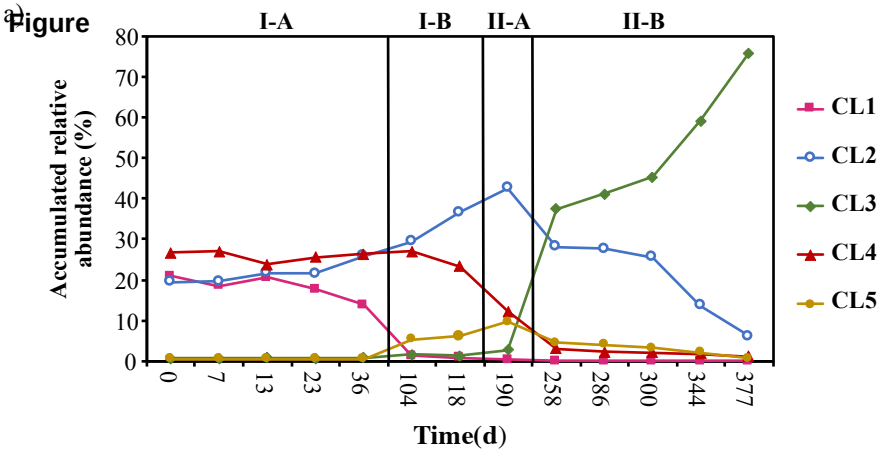

I-A
I-B
II-A
II-B
8
7
6
5
4
3
2
1
0
(g L-1)
0
50
100
150
200
250
300
350
400
Time (days)
MLTSS
MLVSS

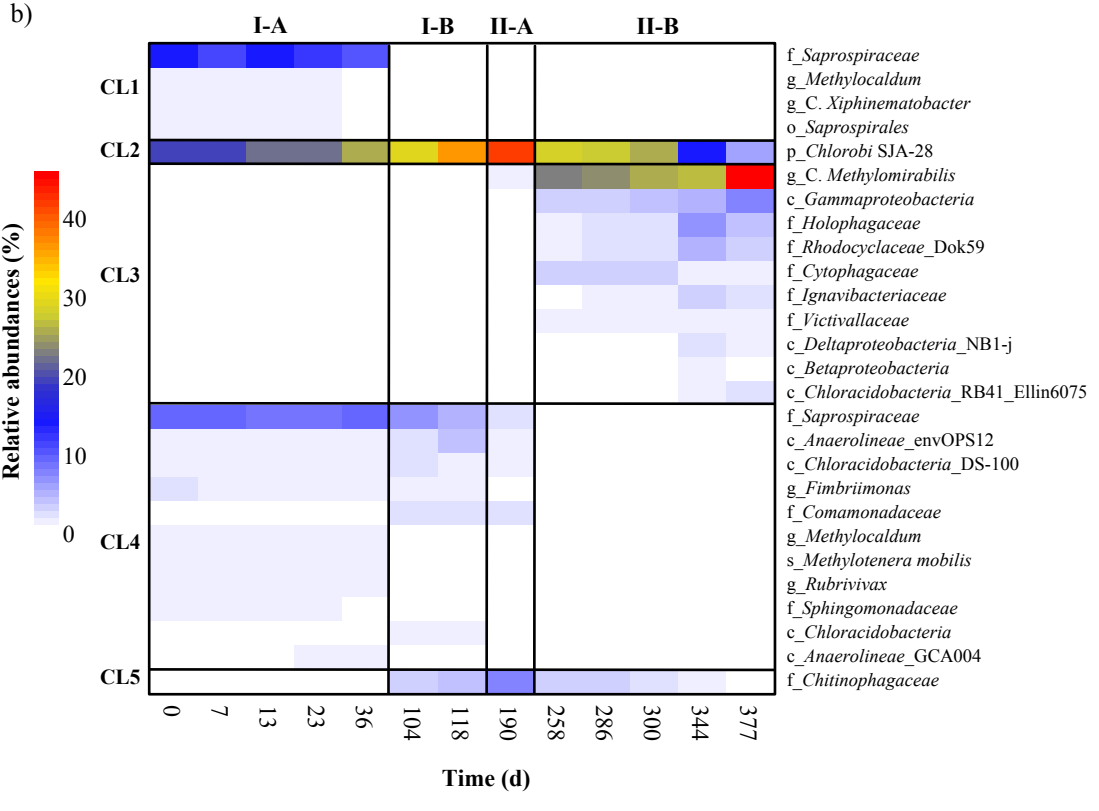

a)
b)
c)
d)


e)
I-A
I-B
II-A
II-B
Relative abundance (%)
0
10
20
30
40
50
60
70
80
90
100
0
7
13
23
36
104
118
190
256
286
300
344
377
Time (d)
Bacteroidetes
Chlorobi
Proteobacteria
Acidobacteria
Verrucomicrobia
Chloroflexi
Armatimonadetes
Planctomycetes
Actinobacteria
Nitrospirae
Gemmatimonadetes
Lentisphaerae
NC10
Minor phyla

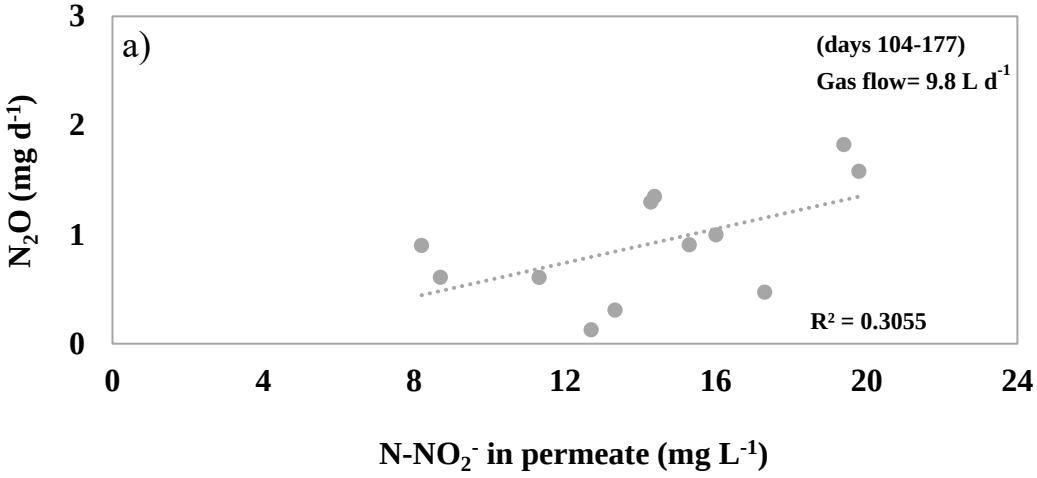

f)
I-A
I-B
II-A
II-B
Relative abundance (%)
0
10
20
30
40
50
60
70
80
90
100
0
7
13
23
36
104
118
190
256
286
300
344
377
Time (d)
Saprospiraceae
Chlorobi (SJA-28_unk)
Methylococcaceae
Comamonadaceae
Fimbriimonadaceae
Chloracidobacteria (DS-100_unk)
Saprospirales_unk
Chitinophagaceae
Anaerolineae (envOPS12_unk)
Rhodocyclaceae
Chloracidobacteria (Ellin6075_unk)
Deltaproteobacteria (NB1-j_unk)
Methylocystaceae
Cytophagaceae
Holophagaceae
Ignavibacteriaceae
Gammaproteobacteria_unk
Methylomirabiliaceae
Minor families

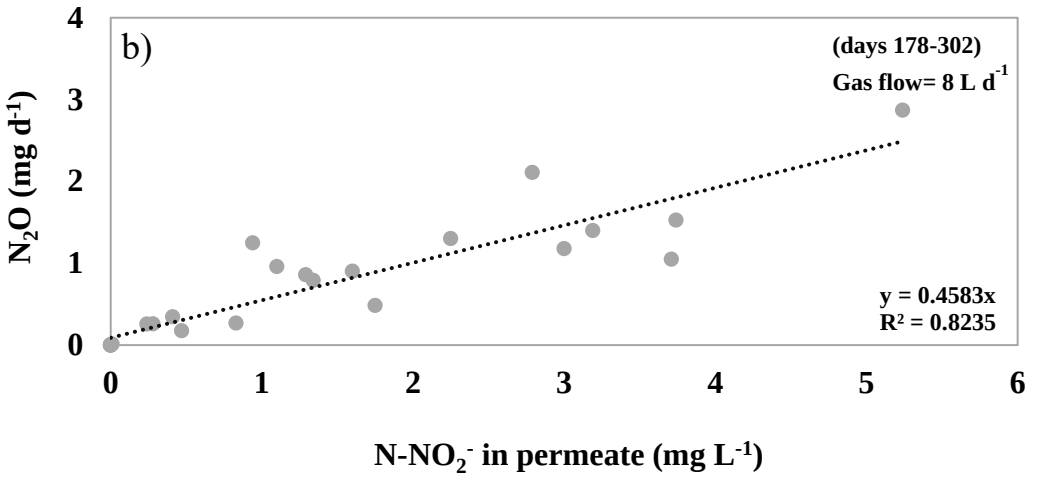
Figure

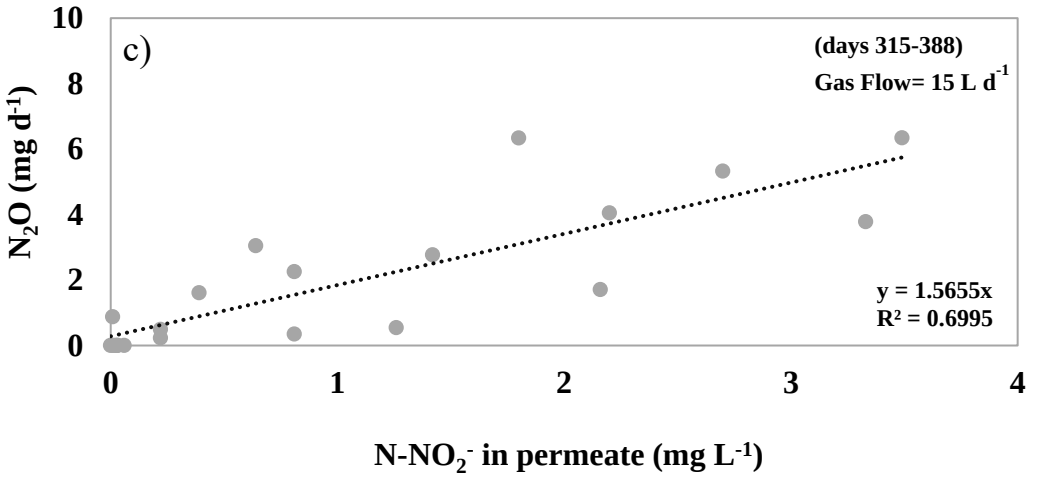

a)
I-A
I-B
II-A
II-B
Accumulated relative abundance (%)
0
10
20
30
40
50
60
70
80
0
7
13
23
36
104
118
190
258
286
300
344
377
Time(d)
CL1
CL2
CL3
CL4
CL5


b)
I-A
I-B
II-A
II-B
CL1
CL2
CL3
CL4
CL5
Relative abundances (%)
0
10
20
30
40
0
7
13
23
36
104
118
190
258
286
300
344
377
Time (d)
f_Saprospiraceae
g_Methylocaldum
g_C. Xiphinematobacter
o_Saprospirales
p_Chlorobi SJA-28
g_C. Methylomirabilis
c_Gammaproteobacteria
f_Holophagaceae
f_Rhodocyclaceae_Dok59
f_Cytophagaceae
f_Ignavibacteriaceae
f_Victivallaceae
c_Deltaproteobacteria_NB1-j
c_Betaproteobacteria
c_Chloracidobacteria_RB41_Ellin6075
f_Saprospiraceae
c_Anaerolineae_envOPS12
c_Chloracidobacteria_DS-100
g_Fimbriimonas
f_Comamonadaceae
g_Methylocaldum
s_Methylotenera mobilis
g_Rubrivivax
f_Sphingomonadaceae
c_Chloracidobacteria
c_Anaerolineae_GCA004
f_Chitinophagaceae

Figure


a)
(days 104-177)
Gas flow= 9.8 L d-1
R² = 0.3055
N2O (mg d-1)
N-NO2- in permeate (mg L-1)
0
1
2
3
0
4
8
12
16
20
24


b)
(days 178-302)
Gas flow= 8 L d-1
y = 0.4583x
R² = 0.8235
N2O (mg d-1)
N-NO2- in permeate (mg L-1)
0
1
2
3
4
0
1
2
3
4
5
6


c)
(days 315-388)
Gas Flow= 15 L d-1
y = 1.5655x
R² = 0.6995
N2O (mg d-1)
N-NO2- in permeate (mg L-1)
0
2
4
6
8
10
0
1
2
3
4