Denitritation in an anoxic granular reactor using phenol as sole organic carbon source

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Abstract

Heterotrophic denitrification of nitrite (denitritation) with phenol as the only organic carbon source was successful achieved in an Upflow Sludge Blanket (USB) reactor with granular biomass. High nitrite removal (over 95 %) and complete biodegradation of phenol were obtained at low COD/N ratio (2.5 ± 0.1) and at high nitrogen and organic loading rates (0.6 ± 0.1 g N L^{-1} d^{-1} and 1.6 ± 0.3 g COD L^{-1} d^{-1} at 30 ºC, respectively). The granulation remained stable throughout the operational period with an average granule size of 0.9 ± 0.1 mm and 95 % of the biomass with a diameter higher than 0.2 mm. The denitrifying granular biomass was also able to use nitrate as electron acceptor after one year consuming exclusively nitrite. However, the granular biomass was not able to use *o*-cresol as electron donor although phenol and nitrite degradations were not affected by the presence of *o*-cresol and the granules remained stable. Most of the genera identified in the granules after one year operation of the anoxic USB reactor

were denitrifying bacteria able to use nitrite and nitrate as electron acceptors and aromatic compounds as electron donors. The results presented in this study open the possibility of designing a new process for the treatment of complex wastewaters contaminated with aromatic compounds.

Keywords: granules; heterotrophic denitrification; phenolic compounds

1. Introduction

Industries from fertilizer, petrochemical, pharmaceutical and chemical sectors produce wastewaters containing: (i) mainly organic compounds, (ii) mainly ammonium or (iii) both, ammonium and organic compounds (see Table S1 in supporting information for more details). Aromatic compounds, with toxic/recalcitrant properties, are the most frequently compounds found in these wastewaters [1, 2]. Both, physico-chemical and biological processes can be applied to treat these wastewaters. When applying physico-chemical process high energy costs, incomplete degradation and secondary pollutants are usually obtained [3]. Biological processes can satisfactorily overcome some of the disadvantages of physico-chemical processes. Technologies based on flocculent biomass, such as activated sludge systems, are the main biological processes implemented at full-scale, however its practical application for treating complex industrial wastewaters is rather limited because activated sludge systems are known to be inhibited by aromatic compounds [3]. To overcome the inhibition caused by organic compounds, a promising alternative to activated sludge systems is the application of reactors with aerobic granular biomass [4]. The application of aerobic granules allows

retaining slow growing microorganisms and protects them from high concentrations of pollutants due to the diffusion gradients generated along the granule [4] that favour the gradual adaptation of microorganisms to stressing conditions.

When treating ammonium contaminated wastewater, Biological Nitrogen Removal (BNR) processes appear as the technology of choice, but it can be inhibited by the presence of aromatic compounds [2]. Moreover, conventional BNR processes need elevated aeration (high energy costs) during nitrification and high organic carbon requirements during denitrification [5]. However, BNR processes via nitrite are recognized as cost-effective alternative compared to conventional nitrification-denitrification processes, due to the significant reduction of aeration and external carbon source requirements [5].

A two-stage process with granular biomass could be applied to satisfactorily deal with the treatment of wastewaters composed by ammonium and aromatic compounds. In this scheme, the first stage simultaneously performs nitritation (bio-oxidation of ammonium to nitrite) and aerobic biodegradation of aromatic compounds in a granular airlift reactor [6, 7] producing a suitable effluent without aromatic compounds either for: (i) heterotrophic denitrification of nitrite (denitritation) (the ammonium in the influent is completely oxidized to nitrite) or (ii) anammox process (half of the ammonium in the influent is oxidized to nitrite).

If the second stage consists of denitritation (bio-reduction of nitrite to nitrogen gas), an organic carbon source is required. Readily biodegradables substrates (such as methanol and acetate) can be applied [8-10], however, high operational costs take place. In this sense, alternative organic carbon sources should be used. These carbon sources could be effluents from the same industries stated before containing mainly aromatic compounds.

However, both substrates, nitrite and aromatic compounds, can produce inhibition by substrate of the denitritation process [11, 12]. The quantification of this inhibition through a kinetic model could be of interest for the scale-up of the process. Therefore, the development of a denitritation process with aromatic compounds as organic carbon source is of paramount relevance to achieve an efficient, cost-effective and environmental friendly treatment of the effluents generated by these industries. Hence, the main objective of this study is to demonstrate that denitritation with aromatic compounds as the sole organic carbon source can be satisfactorily achieved in an anoxic Upflow Sludge Blanket (USB) reactor with granular biomass. To this end, the maximum removal capacity of the USB reactor, the stability of granular biomass at long-term operation, kinetics of substrate inhibition and the microbial diversity and its function in the granules will be assessed.

2. Materials and methods

2.1. Reactor

Continuous experiments were conducted in an USB reactor made of glass (2.2 L of effective liquid volume). The column part of the USB reactor has a height of 45 cm and an internal diameter of 64 cm, which represented 1.75 L. A conventional solid-liquid-gas separator was on the top of the reactor. The reactor was equipped with pH (Crison pH 5333) and temperature (Crison Pt1000) probes that were connected to a data monitoring system (Crison Multimeter 44). A Programmable Logic Controller (PLC) coupled to a Supervisory Control And Data Acquisition (SCADA) system controlled temperature and feeding pump. Temperature was fixed at 30.0 ± 0.5 °C using a

temperature controller coupled with a belt-type heating device (Horst, Germany). pH was continuously monitored but it was not controlled, ranging between 7.8-8.2. The wastewater was continuously fed to the bottom of reactor with the use of a membrane pump (Milton Roy LMI) through and a diffuser to produce homogeneous distribution of the wastewater.

2.2. Inoculum

USB reactor was inoculated with granular sludge from a full-scale internal circulation reactor treating an industrial wastewater through anaerobic digestion. The characteristics of these granules were: sludge volumetric index at 30 min (SVI_{30}) of 8.0 ± 0.5 mL g^{-1} TSS (total suspended solids), SVI_{30}/SVI_5 of 1.0, average granule size of 1.1 ± 0.1 mm and 87 % of biomass with a diameter higher than 0.2 mm. One liter of granular biomass was added to the reactor at the beginning of the reactor operation.

2.3. Wastewater composition

USB reactor was fed continuously with a synthetic wastewater. Nitrite or nitrate and phenol or phenol plus *o*-cresol were used as electron acceptors or electron donors, respectively and their concentrations varied depending on the applied conditions. The composition of the micronutrients in the synthetic wastewater was maintained constant along the whole experimental period and was composed as follows (expressed as mg L^{-1}): 150 KH_2PO_4; 5 $MgSO_4x7H_20$; 0.5 $FeCl_3x6H_2O$; 0.5 KCl; 0.05 $CaCl_2x2H_2O$ and 100 yeast extract. The conductivity and pH of the influent were 1.3 ± 0.2 mS cm^{-1} and 7.1 ± 0.2, respectively. The synthetic influent was maintained at 4 °C to avoid changes in the wastewater composition.

2.4. Operational conditions applied to the anoxic USB reactor

The anoxic USB reactor was operated during 400 days under 3 different conditions. Throughout the whole operation, the stoichiometric COD/N ratio for denitrification was fed to promote denitrification instead of anaerobic metabolic pathways:

i) Heterotrophic denitritation using phenol as sole organic carbon source (from day-0 to day-328). During this period, nitrite concentration in the influent was set at 70 mg N-NO_2^- L^{-1} except for 30 days when it was increased to 110 mg N-NO_2^- L^{-1}. The applied COD/N ratio was always 2.5, which resulted from the stoichiometric ratio determined by the denitritation reaction with phenol as organic carbon source, including biomass production (Equation 3).

$$1.33NO_2^- + 0.21C_6H_6O \rightarrow 0.05C_5H_7NO_2 + 0.64N_2 + 1.02CO_2 + 0.18H_2O + 0.56OH^-$$

Equation 3

ii) Heterotrophic denitrification using phenol as sole organic carbon source (from day-328 to day-334). Nitrate concentration in the influent was set at 70 mg N-NO_3^- L^{-1} and the applied COD/N ratio was 4.1, which resulted from the stoichiometric ratio determined by the denitrification reaction with phenol as organic carbon source, including biomass production (Equation 4).

$$1.57NO_3^- + 0.38C_6H_6O \rightarrow 0.12C_5H_7NO_2 + 0.68N_2 + 1.69CO_2 + 0.26H_2O + 0.92OH^-$$

Equation 4

iii) Heterotrophic denitritation using phenol and *o*-cresol as organic carbon sources (from day-350 to day-400). Between days 350 to 366, nitrite, phenol and *o*-cresol concentrations in the influent were set at 70 mg $N\text{-}NO_2^-$ L^{-1}, 32 mg L^{-1} and 32 mg L^{-1}, respectively. Therefore, the applied COD/N ratio was maintained in 2.5. Later, from day-366 until day-378, nitrite was increased to 100 mg $N\text{-}NO_2^-$ L^{-1} and phenol and *o*-cresol remained at 32 mg L^{-1} each one. In this sense, the applied COD/N ratio decreased to 1.7. Finally, the operational conditions were reestablished to 70 mg $N\text{-}NO_2^-$ L^{-1}.

2.5. Samples and analytical methods

Samples were regularly withdrawn from the influent and the effluent and filtered through 0.20 μm syringe filter driven unit from Milipore® provided with a high-density polyethylene housing and membrane of hydrophilic Durapore® prior to analysis. Nitrate and nitrite concentrations were analyzed with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation), which performs ion analyses using suppressed conductivity detection.

Phenol and *o*-cresol were measured by High Performance Liquid Chromatography (HPLC), using a UltiMate 3000 (Dionex Corporation) with a Agilent Zorbax SB-C18 (4.6 mm x 100 mm x 3.5 μm) column and a UV detector set at 254 mm, the flow rate was 1.875 mL min^{-1} and the column temperature was maintained at 30 °C. The mobile phases were acidified water (ultrapure water containing H_2SO_4 at pH 1.41) and HPLC-grade methanol following a gradient elution. The gradient started from 100 % of acidified water and progressively changed to 50:50 v/v of water:methanol in 18 min,

then it remained isocratic until 20 min. The injection volume was 20 µL and the maximum pressure in the column was approximately 290,000 hPa.

TOC was measured with an OI Analytical TOC Analyzer (Model 1020A) equipped with a non-dispersive infrared (NDIR) detector and a furnace maintained at 680 °C. Chemical oxygen demand (COD) was calculated from the TOC experimental data using the theoretical relationship between COD and TOC of the combustion reaction for each organic compound. Total and volatile suspended solids (TSS and VSS, respectively) were determined in the reactor and in the effluent, according to the Standard Methods 2540 D and 2540 E [13].

Nitrous oxide (N_2O) emissions were measured by collecting off-gas grab samples from separator at the top of the USB reactor using a tube connected to a gas sampling bag. Each off-gas sample was continuously collected during 24 h. Four off-gas grab samples were collected during a week and an average N_2O concentration was used for calculating the N_2O emission. N_2O concentration (expressed in ppmv) was measured with gas chromatography (Agilent Technologies 6890N Network GG System) equipped with a semi-capillary (HP-PLOT Q 30 m x 0.53 mm x 40.0 µm) column (Agilent Technologies) and electron capture detector. The temperature of the injector, detector and oven were set at 120, 345 and 60 °C, respectively. 500 µL were injected and analyzed during 4 minutes. N_2O emissions were calculated based on the relation between the N_2O produced to the nitrite removed (g $N\text{-}N_2O$ g^{-1} $N\text{-}NO_2^-$) as follows (equation 1):

$$\%\ N_2O\ \text{emission} = \frac{C_{N-N_2O\ gas}\ x\ Q_{gas}}{NRR\ x\ V} \times 100 \qquad \text{Equation 1}$$

Where $C_{N\text{-}N_2O\ gas}$ is the N_2O concentration (g N-N_2O L^{-1}), Q_{gas} is the gas flow rate going out the reactor (L d^{-1}), NRR is the N-nitrite removal rate (g N-NO_2^- L^{-1} d^{-1}) and V is the reaction volume (L).

Nitrous oxide concentration was determined using equation 2.

$$C_{N-N_2O\ gas} = C_{N_2O\ gas}\ x\left(M_{VN_2O}\right)^{-1} \times 1x10^{-6} \times n \times MW_N \qquad \text{Equation 2}$$

Where $C_{N_2O\ gas}$ is the N_2O concentration (ppm_v=mL m^{-3}), M_{VN_2O} is the N_2O molar volume (24.15 L mol^{-1} at 25 °C and 1 atm), $1x10^{-6}$ is the unit conversion factor from mL to m^3, n is the mol N mol^{-1} N_2O ratio (2) and MW_N is the molecular weight of nitrogen (14 g mol^{-1}).

The granular biomass was characterized in terms of sludge volumetric index (SVI), size and morphology. SVI was determined using the 2710 D procedure described in Standard Methods [13]. The distribution of the granules size was measured by a laser particle size analysis system (Mastersizer 2000, Malvern instruments). Granules morphology was assessed by image analysis with an optical microscope (Zeiss Axioskop equipped with an iAi Protec video camera) and by Scanning Electron Microscope (SEM) with an EVO MA 10 scanning electron microscope (Zeiss). The methodology for SEM can be found elsewhere [14].

2.6 Microbial diversity analysis

2.6.1 DNA Extraction and Sequencing

Granular biomass was taken from the inoculum (d-0) and from the anoxic USB reactor at day-325 (d-325) and their DNA was extracted and purified using the PowerBiofilm™ DNA Isolation Kit (MoBio Laboratories, USA) following the manufacturer protocol, with the only exception of solution BF3 that was added to 200 mL instead of the 100 mL recommended by the manufacturer. The quality and quantity of extracted DNA were measured by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). All DNA samples were adjusted to 25 ng mL^{-1} for pyrosequencing. Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by Research and Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V4-V6 were targeted using the primer pair 939F-1492R.

2.6.2 Biodiversity analysis and phylogenetic classification

The forward and reverse reads were merged together using the PEAR Illumina paired-end read merger [15], sequence reads were then sorted by length from longest to shortest and prefix dereplication and clustering at a 4% divergence was performed using the USEARCH algorithm [16]. Following, the clusters were classified into operational taxonomic units (OTUs) using the UPARSE OTU selection algorithm [17]. Chimera checking was performed using the UCHIME chimera detection software executed in *de novo mode* [18]. The representative sequences reads were mapped to their corresponding nonchimeric cluster using the USEARCH global alignment algorithm [16].

In order to determine the identity of each remaining sequence, the sequences were quality checked and demultiplexed using the denoised data generated previously. Sequences with fewer than 276 bps were removed. Sequences that passed the quality

control screening were then clustered into OTUs using the UPARSE algorithm [17]. Each of the original reads was then assigned back to their OTUs using the USEARCH global alignment algorithm [16]. The centroid sequence from each cluster was run against the USEARCH global alignment algorithm along with a database of high quality sequences derived from NCBI and maintained by Research and Testing Laboratory. For each OTU, the top six matches from the high quality database were kept and confidence values were assigned to each taxonomic level by taking the number of taxonomic matches that agree with the best match at that level and dividing that by the number of high quality sequence matches that were found. Each OTU was then assigned taxonomic information using the lowest common taxonomic level whose confidence value was above 51%. OTUs that received no matches against the high quality sequences were identified as "no hit". After resolving the number of sequences per OTU, the percentage of each organism was individually calculated for each sample. Data obtained provided relative abundance information within and among individual samples. Relative abundances of reads were calculated by taxonomic level for each library (d-0 and d-325). Values represent the percentage of reads of sequences obtained at each taxonomic identity (according to the degree that of similarity described above) within the total set of readings from the library. In bacteria, the rRNA operon is frequently found in multiple copies (1-15) [19]. Therefore, the community structure can be biased as one may obtain sequences with a lesser abundance but high number of reads (multiples copies of the 16S gene), or a higher abundance but low number of reads (single copy of the 16S gene). To remove this bias, the relative abundances of reads by taxonomic level for each library have been normalised by the average number

of copies of the rRNA operon of each taxonomic level using the database freely available at https://rrndb.umms.med.umich.edu/.

Indices of biological diversity were calculated for d-0 and d-325 libraries (Table S2 and Figures S1 and S2 of the supporting information) indicating all libraries were comparable in terms of abundance percentages and that good coverage of diversity was reached.

2.7. Haldane kinetic model

Using the data gathered from the USB reactor operation, inhibition by substrate (nitrite and phenol) was described with a Haldane kinetic model:

$$r = \frac{r_{MAX} \times S}{K_S + S + \frac{S^2}{K_I}} \qquad \text{Equation 5}$$

Where r is the removal rate (g L^{-1} d^{-1}), r_{MAX} is the maximum removal rate (g L^{-1} d^{-1}), S is the substrate concentration (mg L^{-1}), K_S is the affinity constant (mg L^{-1}) and K_I is the inhibition constant (mg L^{-1}). Haldane kinetic model was fitted to the experimental data using the SigmaPlot®11.0 software (Systat Software Inc. California, USA).

Furthermore, two other parameters can be calculated for a Haldane kinetic model:

$$r_{CI} = \frac{r_{MAX}}{1 + 2 \times \sqrt{\frac{K_S}{K_I}}} \qquad \text{Equation 6}$$

$$S_{CI} = \sqrt{K_S \times K_I} \qquad \text{Equation 7}$$

Where r_{CI} is the critical rate constant or the practical maximum removal rate and S_{CI} is the substrate concentration at which r_{CI} is attained.

2.8. Growth yields calculation

Observed growth yields were calculated at different steady state conditions. Each observed growth yield was calculated as the variation of the biomass in the reactor plus the biomass loss in the effluent divided by the total consumption of organic matter in the same period.

3. Results

3.1. USB reactor performance using nitrite as electron acceptor and phenol as the sole organic carbon source

USB reactor performance during the first 328 days is presented in Figure 1. The operation of the USB during this period can be divided in four periods: (I) start-up and acclimation (days 0-150), (II) steady state conditions (days 150-210), (III) increase of the nitrogen loading rate (NLR) to determine the maximum capacity of the reactor (days 210-280) and (IV) return to steady state conditions (days 280-328). During the first 20 days of period I, low NLR and organic loading rates (OLR) were applied (0.1 g N L^{-1} d^{-1} and 0.3 g COD L^{-1} d^{-1}, respectively) with an upflow velocity (V_S) of 0.05 m h^{-1}, a hydraulic retention time (HRT) of 17 h and a nitrite removal lower than 80%. Later, both NLR and OLR were increased up to 0.6 ± 0.1 N g L^{-1} d^{-1} and 1.6 ± 0.3 g COD L^{-1} d^{-1} and consequently, the V_S increased to 0.25 m h^{-1} and the HRT decreased to 2.6 h.

Since then, nitrite was almost completely removed. On the contrary, the phenol removal was partial during the start-up period (10 to 70%) and the COD removal increased from 20 up to 80% in this first period.

In period II, USB reactor reached steady state conditions since nitrite and phenol were almost completely removed. Moreover, there was not accumulation of intermediates of the anoxic biodegradation pathway of phenol as demonstrated by a COD removal close to 100%. The COD/N ratio consumed during this period was 2.5 ± 0.1, which is the stoichiometric value for denitritation with phenol. In these conditions, the achieved N-nitrite removal rate (NRR = 0.6 ± 0.1 g N L^{-1} d^{-1}) may not be the maximum capacity of the anoxic USB reactor since the biomass was limited by nitrite in this period.

In period III, both NLR and OLR were increased up to 0.9 ± 0.2 g N L^{-1} d^{-1} and 2.1 ± 0.8 g COD L^{-1} d^{-1}, respectively, to determine the maximum removal capacity of the granular reactor. Immediately, nitrite and phenol were accumulated and nitrogen and COD removal efficiencies decreased to 66 ± 18 % and 53 ± 22 %, respectively. The NRR achieved in these conditions was again 0.6 ± 0.1 g N L^{-1} d^{-1}. Considering that the granules were not limited by nitrite in this period, this NRR can be considered the maximum denitrifying capacity of the anoxic USB reactor at 30 ºC with nitrite as electron acceptor and phenol as the sole organic carbon source. In period IV, NLR and OLR were reduced to 0.6 ± 0.1 g N L^{-1} d^{-1} and 1.6 ± 0.3 g COD L^{-1} d^{-1}, respectively from day-280 onwards to check the robustness of the anoxic USB reactor. In few days, the reactor achieved steady state conditions with the similar consumed COD/N ratio (2.4 ± 0.6) and NRR (0.6 ± 0.1 g N L^{-1} d^{-1}) than that of period II.

The average observed growth yield with nitrite as electron acceptor and phenol as electron donor was calculated with data of steady state conditions as 0.19 ± 0.08 g VSS g^{-1} COD.

Nitrous oxide (N_2O) emissions were measured on days 281-299 and on days 320-324 during the operation of the anoxic USB reactor with nitrite and phenol. During days 281-299, low nitrite and phenol concentrations in the effluent were observed (3 mg N L^{-1} and 0 mg phenol L^{-1}, respectively), while for days 320-324, higher concentrations of nitrite and phenol were recorded (29 mg N L^{-1} and 6 mg phenol L^{-1}, respectively). The obtained values, expressed as N_2O produced by nitrite removed, were 0.01 ± 0.01 and 0.03 ± 0.01 %, for days 281-299 and days 320-324, respectively.

Finally, a Haldane kinetic model was fitted to the following data: (i) different NRR achieved at several nitrite concentrations (Figure 2A) and (ii) different ORR achieved at several phenol concentrations (Figure 2B) in the USB reactor during the non-steady state conditions. The achieved kinetic parameters are presented in Table 1.

3.2. USB reactor performance using nitrate as electron acceptor

The capacity of the granular biomass to change the electron acceptor after a long-term operation with nitrite was evaluated by modifying the influent composition replacing nitrite by nitrate from day-328 to day-334 (data do not shown). Nitrate concentration and COD/N ratio in the influent, NLR and OLR were fixed at 70 mg N L^{-1}, 4.1, 0.6 g N L^{-1} d^{-1} and 2.4 g COD L^{-1} d^{-1}, respectively. Nitrate was almost completely consumed from the beginning (4 ± 1 mg N L^{-1} in the effluent) and low nitrite concentration was detected in the effluent (3 ± 1 mg N L^{-1}). Therefore, the nitrogen removal was 90 ± 1 %. For the case of COD removal, it was remained at 77 %. The consumed COD/N ratio

was 3.4 ± 0.5. Moreover, N_2O emissions were also measured in this period, achieving a value of 0.01 ± 0.01 %.

3.3. USB reactor performance using phenol and *o*-cresol as organic carbon sources

The capacity of the granular biomass to use a second aromatic compound as organic carbon source was evaluated by adding *o*-cresol to the influent on days 350 to 400 (Figure 3). Before the *o*-cresol addition, USB reactor was set-up to stable operational conditions treating only nitrite and phenol. From day-350, phenol and *o*-cresol were fed to the USB reactor at 32 mg L^{-1} each one. NLR and OLR were kept at the same values than in periods II and IV (0.6 g N L^{-1} d^{-1} and 1.6 g COD L^{-1} d^{-1}, respectively). Nitrite and phenol were completely consumed, but *o*-cresol removal was only ca. 15 %. Therefore, nitrite removal was close to 96 % and COD removal around 46 %. Nitrite concentration in the influent was increased from 70 to 100 mg N L^{-1} from day-366 to day-378 to evaluate if the low *o*-cresol removal could be due to a nitrite limitation. Immediately, nitrite was accumulated in the reactor, N-removal decreased to 54%, phenol removal remained close to 100%, but *o*-cresol removal did not improve. NRR in these conditions was 0.5 g N L^{-1} d^{-1}, corroborating that the maximum capacity of this system was around 0.5-0.6 g N L^{-1} d^{-1}.

Finally, NLR was again decreased to 0.6 g N L^{-1} d^{-1} from day-378 onwards to evaluate the recovery of the anoxic USB reactor. In 10 days, N-removal increased to 97% with almost complete phenol removal but still low *o*-cresol removal. Finally, the average observed growth yield with nitrite as electron acceptor and phenol and *o*-cresol as electron donor was calculated with data of steady state conditions as 0.22 ± 0.06 g VSS g^{-1} COD.

3.4. Morphological characteristics of the granular biomass

The morphological characteristics of the granular biomass were evaluated throughout the USB reactor operation with nitrite as electron acceptor and phenol as the sole organic carbon source. Total and volatile solids concentrations and VSS/TSS ratio in the reactor and total solids concentration in the effluent remained stable throughout the operational period at 41 ± 7 g TSS L^{-1}, 15 ± 2 g VSS L^{-1}, 0.36 and 25 ± 5 mg TSS L^{-1}, respectively. SVI_5 and SVI_{30} had the same low value (15 ± 1 mL g^{-1} TSS) throughout the operational period and consequently, its ratio was always 1.

Figure 4 shows the average granule size and the percentages of particles with diameters higher and lower than 0.2 mm. Particles with diameter higher than 0.2 mm are considered as granules while particles with diameter lower than 0.2 mm are considered flocs [19]. The inoculum had an average granule size of 1.1 ± 0.1 mm with a high percentage of granules (87 %). During the start-up and acclimation period, both the average diameter (0.31 ± 0.02 mm) and the percentage of granules (46 %) decreased compared to the inoculum.

Moreover, a progressive change in the appearance and the colour of the sludge was observed. Granules aspect went from black anaerobic granules of the inoculum to brownish denitrifying granules, once the biomass was adapted to anoxic conditions (see Figure S3 of the supporting information).

The average diameter and the percentage of granules increased to 0.67 ± 0.05 mm and 87 % respectively, once steady state conditions of nitrite and phenol removal were achieved in the anoxic USB reactor (days 150-210). These morphological characteristics remained stable throughout the period, in which NLR and OLR increased

(days 210-280). In the last period, at steady state conditions (days 280-328), both the average diameter and the percentage of granules increased up to 0.80 ± 0.08 mm and 95 %, respectively.

3.5. Microbial characterization of the granular biomass

Next-generation sequencing (MiSeq Illumina) was used to evaluate the microbial community changes during the acclimation process of the anaerobic granular biomass to denitritation with phenol. Two amplicon libraries, namely d-0 and d-325 were constructed with biomass samples obtained from day-0 (inoculum) and day-325 (steady state conditions). After quality analysis and removal of low quality sequences, 41533 sequences were annotated, corresponding to 32749 high-quality V4-V6 tags of the 16S rRNA-gene in library d-0 and to 8784 in d-325, with an average length of 510 bps per sequence for both libraries. Figure 5 shows the results of bacterial diversity at class and genus levels for the inoculum used in the start-up (d-0) and the denitrifying granular biomass at day-325 (d-325), respectively.

About the anaerobic granular biomass used as inoculum, the most abundant group at class level (54 %) was classified as unknown i.e. they were probably uncultured bacteria. The representative sequence of this OTU was ran against BLAST and this sequence matched with uncultured bacterium clone (GenBank GU389591.1) [20] and uncultured bacterial gene (GenBank AB266945.1) [21] in anaerobic digesters treating high-strength COD inlets. Other significant groups at class level were: Deltaproteobacteria (11 %), Clostridia (5 %) and Cytophagia (5 %), as shown Figure 5A. These four classes represented the 75 % of the analyzed DNA sequences. At genus level (Figure 5B), the predominant group (59 %) was also unknown. Other significant

groups at genus level were: *Syntrophobacter* (10 %), *Cythophaga* (4 %), *Syntrophus* (3 %), *Clostridium* (2 %), *Spirochaeta* (2 %), *Longilinea* (2%) and *Bacteroides* (1 %) (Figure 5B), representing the 24 % of the analyzed DNA sequences.

Regarding denitrifying granular biomass at day-325, Ignavibacteria was the most abundant group at class level (50 %), followed by Betaproteobacteria (37 %) and Chloroflexi (11 %), as shown in Figure 5A. These three classes represented the 98 % of the analyzed DNA sequences. At genus level, *Ignavibacterium* was the predominant group (54 %), followed by *Denitratisoma* (30 %), *Chloroflexus* (7 %), *Thaurea* (3 %) and *Aquincola* (3 %) (Figure 5B), representing the 97 % of the analyzed DNA sequences.

4. Discussion

4.1. Maximum nitrogen removal capacity and nitrous oxide emissions

Denitrification in granular reactors with nitrate as electron acceptor and aromatic compounds as electron donors has been previously reported [22-24]. But, to the best of our knowledge, this is the first study describing the successful performance of an anoxic granular reactor with nitrite as electron acceptor and phenol as electron donor.

Nitrate removal rates (NRR) reported in anoxic USB reactors with nitrate as acceptor electron range from 0.20 g N L^{-1} d^{-1} (with phenol and 3,4-dimethylphenol as organic carbon sources, [22]) to 0.35 g N L^{-1} d^{-1} (with phenol, cresols and dimethylphenols as organic carbon sources, [24]). The NRR achieved in this study with nitrite and phenol (0.6 g N L^{-1} d^{-1}) is about twofold higher than those reported values. The reasons for this higher NRR in this study could be: (i) denitrification and methanogenesis took place

simultaneously in the reported anoxic USB reactors while, in this study, only denitritation took place. As methanogenic and denitrifying bacteria compete for the same organic substrates, if both processes take place simultaneously in a USB reactor, the maximum NRR feasible in that reactor would be limited, (ii) the upflow velocity used in this study (0.25 m h^{-1}) was significantly higher than that used in the reported anoxic USB (0.02 m h^{-1} [23]; 0.06 m h^{-1} [24]). This means that the substrate external and internal mass transfer limitations were smaller in the USB reactor of this study than that the reported in the literature. Consequently, the nitrate removal rates reported in the literature could be also conditioned by substrate mass transfer limitations.

The nitrous oxide emissions measured in the anoxic USB reactor were 0.02 % (nitrite as electron acceptor) and 0.01% (nitrate as electron acceptor). These values are similar than the reported in other denitrifying reactors (0.005-0.5%) [25, 26]. Other authors reported higher emissions values than those reported here for denitrifying reactors but these emissions seem to be influenced by operational factors such as: (i) presence of dissolved oxygen (DO) [27] or (ii) high nitrite concentration [28] in the denitrifying reactor. In this study, the DO concentration in the anoxic USB reactor was always zero and the nitrite concentration accumulated during the N_2O emissions measurements did not excess 30 mg N-NO_2^- L^{-1}. Consequently, an anoxic USB reactor treating nitrite and/or nitrate with phenol would not represent a significant source of N_2O emissions.

4.2. Organic matter consumption and biomass production

The consumed COD/N ratio was determined twice at steady state conditions in the anoxic USB reactor. Both values, 2.5 ± 0.1 and 2.4 ± 0.6, were similar to the stoichiometric value for denitritation with phenol as the sole organic carbon source.

This fact confirmed that the main biological process taking place in the anoxic USB reactor was denitritation. Moreover, the consumed COD/N ratio of this study confirms the significant reduction of organic matter consumption in an anoxic reactor if nitrite is used as electron acceptor instead of nitrate.

Also, the use of aromatic compounds as carbon source decreases the excess sludge production. This could be translated in savings in the operation of the wastewater treatment because the quantity of sludge that has to be disposed off is smaller than with other carbon sources. This fact is confirmed by the lower observed growth yields achieved with phenol (0.19 ± 0.08 g VSS g^{-1} COD) and with phenol and *o*-cresol (0.22 ± 0.06 g VSS g^{-1} COD) than the achieved with more biodegradable substrates (0.27-0.42 g VSS g^{-1} COD [29]). These low observed growth yields and consumed COD/N ratio mean that the biomass production in the anoxic USB reactor was very low, which can be considered as an advantage for the stability of the granular biomass.

4.3. Inhibition by substrate

Previous studies with anoxic USB reactors reported inhibitory effects of the electron acceptor (nitrate or nitrite) or the electron donor (aromatic compounds) on the denitrification process. For instance, denitrification with nitrate and aromatics was inhibited by accumulation of *m*-cresol [22], while, denitritation with nitrite and methanol was inhibited by accumulation of nitrite [8].

In this study, phenol and nitrite were simultaneously accumulated in the USB during non-steady state conditions (days 0-150 and days 210-280, Figure 1). These episodes produced a decrease of the NRR and the organic removal rate (ORR) and this effect could be understood as an inhibition by both substrates since, nitrite and phenol were

the substrates of the denitritation process. The inhibition by substrate is a well-known process that can be described by several kinetic models, being the most used the Haldane model [30]. In this sense, Figure 2A and Figure 2B show that the experimental data correctly fitted the Haldane kinetic model, with the parameters presented in Table 1. Firstly, the practical maximum removal rates (r_{Cl}) for nitrite and phenol agreed with the maximum NRR and ORR achieved in the USB. Secondly, the inhibitory effect of both, nitrite and phenol was demonstrated by the values of the inhibition constants (10 mg N L^{-1} for nitrite and 24 mg L^{-1} for phenol). These values indicate that the denitritation process with phenol as the sole organic carbon source is strongly affected by the accumulation of both substrates. Consequently, the performance of this process in a continuous reactor, such as USB reactor, is recommended to avoid the peak of substrates that occurs in batch reactors at the beginning of a cycle. Finally, the low values of both affinity constants (5 mg N L^{-1} for nitrite and 0.4 mg L^{-1} for phenol) indicate that the denitrifying granular biomass had a high affinity for both substrates, which means that very low concentrations of both substrates in the effluent can be achieved with the proper operation of a continuous reactor.

4.4. Stability of the granular biomass

One of the most important issues that have to be checked to demonstrate the feasibility of a wastewater treatment based on granular biomass is the long-term stability of the morphological characteristics of the granules. The anoxic granular USB reactor was operated for 400 days and throughout this period the morphological characteristics of the granular biomass were recorded to track possible changes (Figure 4). The granules presented a smooth and regular shape indicating a possible absent or very low content

of filamentous microorganisms, as confirmed by the SEM images (Figure S4 of the supporting information).

The inoculum was a mature anaerobic granular biomass with excellent settleability, close to 1 mm of average diameter and 90% of granules. During the acclimation period to anoxic conditions, the characteristics of the granules worsened, 0.3 mm of average diameter, 60% of granules and a wide range of size distribution (Figure 4) was obtained. This was probably due to the change of the microbial population that took place throughout this period (see section 4.5). However, after achieving stable denitrifying conditions in the USB reactor, the characteristics of the granules progressively improved to a stable and mature denitrifying granular biomass with 0.8 mm of average diameter and more than 90% of granules (Figure 4). It is well-known that a high shear force assisted the formation of compact and dense granules by stimulating the production of extracellular polymeric substances [31]. In the case of a USB reactor, this shear force is applied through the upflow velocity. Consequently, the stability at long-term of the anoxic granules of this study can be related to the upflow velocity applied in the USB reactor (0.25 m h^{-1}), which was significantly higher than that reported in other anoxic USB reactors [23, 24].

An additional advantage of the application of USB reactors for denitritation is the good quality of the effluent in term of solids, achieving high biomass concentration inside the reactor (15 ± 2 g VSS L^{-1}) with low TSS concentration in the effluent (25 ± 5 mg TSS L^{-1}). To achieve low concentration of suspended solids in the effluent is a target in the full-scale application of granular reactors [32]. The high quality of the effluent in this study was probably related to the good granulation (granular biomass with 0.8 mm of

average diameter and more than 90% of granules) and good settling capacity of the granules (SVI_5 of 15 ± 1 mL g^{-1} TSS) in the USB reactor.

4.5. Microbial diversity and function in the anoxic USB reactor

The genera identified in the inoculum: *Syntrophobacter*, *Cytophaga, Syntrophus*, *Clostridium* and *Spirochaeta* were anaerobic bacteria usually found in anaerobic reactors producing methane or hydrogen [33] and none of them has been described as denitrifying bacteria able to use nitrite or nitrate as electron acceptors. Moreover, most of these genera were washed out of the USB reactor throughout the acclimation process to denitrifying conditions. Probably, this was the cause of the long acclimation period required after inoculation to achieve a denitrifying granular biomass. The only genus present in the inoculum that remained in the USB reactor and even increased after the acclimation process was *Chloroflexus* (Figure 5B). *Chloroflexus* genus could have an important role in the granulation process because it has been reported in other granular reactors [34].

Ignavibacteria and Betaproteobacteria were the most abundant classes of bacteria in the anoxic granules at day-325 (Figure 5A). Betaproteobacteria contains several microbial groups such as nitrifiers, denitrifiers and other N-cycle related microorganisms. Moreover, denitrification with hydrocarbons appears to be a process that is carried out mainly by Betaproteobacteria [35].

The most abundant genera in the anoxic granules at day-325 were *Ignavibacterium* and *Denitratisoma* (54 and 30 % of the total reads, respectively, Figure 5B), which are denitrifying bacteria recently described [34, 36]. *Ignavibacterium* (54 % of the total reads, Figure 5B) has been described as a versatile group of bacteria able to grow

anoxically [37]. However, *Ignavibacterium* genus seems be able to produce nitrite reductase, nitric oxide reductase and nitrous oxide reductase but not nitrate reductase which means that *Ignavibacterium* genus can grow anoxically using nitrite as electron acceptor but not nitrate [37]. This could be the explanation for the high percentage of this genus in the USB of this study. Moreover, *Ignavibacterium* genus has been found in a bioreactor treating coking wastewater, which contains a high number of aromatic compounds [38]. The second most abundant genus, *Denitratisoma*, is able to use nitrate and nitrite as electron acceptors and several aromatic compounds as electron donors [36, 39]. Among the other genera found in the anoxic USB, *Thaurea* genus (3 % of the total reads) is a well-known group of denitrifying bacteria able use aromatic compounds as electron donors [40]. Consequently, a high percentage of the bacteria in granules at day-325 (87 % of the total reads) were denitrifying bacteria able to use nitrite as electron acceptors and aromatic compounds as electron donors. Moreover, *Denitratisoma* can interchangeably use nitrite and nitrate as electron acceptor, which can explain the fast adaptation of the anoxic USB reactor to consume nitrate after one year exclusively consuming nitrite. Regarding the use of *o*-cresol as electron donor, none of the most abundant genera found in the anoxic USB has been described as able to grow with *o*-cresol.

5. Conclusions

Denitritation using phenol as the sole organic carbon was successfully achieved with granular biomass in an USB reactor.

The nitrite removal rate achieved at steady state conditions was two-fold higher than that reported for denitrification with aromatic compounds.

The obtained granules were able to use nitrate as electron acceptor instead of nitrite, but were not able to use *o*-cresol as electron donor.

The accumulation of nitrite and phenol in the bulk liquid caused inhibition by substrate and this should be avoided for a good performance of the anoxic USB.

The main genera of bacteria in the anoxic granules were *Ignavibacterium* and *Denitratisoma* that have been described as group of bacteria able to reduce nitrite with aromatic compounds as organic carbon source.

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FIGURE CAPTIONS

Figure 1. Performance of the anoxic USB reactor with nitrite as electron acceptor and phenol as the sole organic carbon source. A: Nitrogen Loading Rate (NLR) and Organic Loading Rate (OLR) applied; B: Nitrite, nitrate and phenol concentrations in the influent and effluent of the anoxic USB; C: Nitrogen and COD removal percentages. The operation periods are as follows: I: start-up and acclimation period (days 0-150), II: steady state conditions (days 150-210), III: increase of the nitrogen loading rate (NLR) (days 210-280) and IV: steady state conditions (days 280-328).

Figure 2. Experimental data and kinetic model fitting for denitritation with phenol as sole organic carbon source. A: Nitrite removal rate (NRR) at different nitrite concentrations; B: Organic removal rate (ORR) at different phenol concentrations.

Figure 3. Performance of the anoxic USB with nitrite as electron acceptor and phenol and *o*-cresol as organic carbon sources. A: Nitrogen Loading Rate (NLR) and Organic Loading Rate (OLR) applied; B: Nitrite, phenol and *o*-cresol concentrations in the influent and effluent of the anoxic USB; C: Nitrogen and COD removal percentages.

Figure 4. Evolution of the granulation along the USB reactor performing denitritation using phenol as sole carbon source. A: granule size along the USB reactor operation and B: distribution of the granules size.

Figure 5. Microbial diversity at class (A) and genus (B) level of the anaerobic granular biomass used as inoculum (up) and the anoxic granular biomass at day 325 (down) in the USB reactor. The percentages are referred to the relative abundance of bacteria detected and was defined as the number of sequences affiliated with that taxon divided by the total number of sequences of the library.

Table

Table 1. Kinetic parameters obtained for biomass adapted to denitrify nitrite using phenol as the sole organic carbon source. NRR is the nitrogen removal rate, ORR is the organic removal rate, r_{MAX} is the maximum removal rate, S is the substrate concentration, K_S is the affinity constant, K_I is the inhibition constant, r_{CI} is the critical rate constant or the practical maximum removal rate and S_{CI} is the substrate concentration at which r_{CI} is attained.

Parameter	NRR vs $N\text{-}NO_2^-$		ORR vs phenol	
	Value	Units	Value	Units
r_{MAX}	2 ± 1	g N L^{-1} d^{-1}	2 ± 1	g COD L^{-1} d^{-1}
K_S	5 ± 5	mg N L^{-1}	0.4 ± 0.8	mg phenol L^{-1}
K_I	10 ± 10	mg N L^{-1}	24 ± 25	mg phenol L^{-1}
r_{CI}	0.8 ± 0.6	g N L^{-1} d^{-1}	1.6 ± 0.5	g COD L^{-1} d^{-1}
S_{CI}	7 ± 7	mg N L^{-1}	3 ± 8	mg phenol L^{-1}

Figure

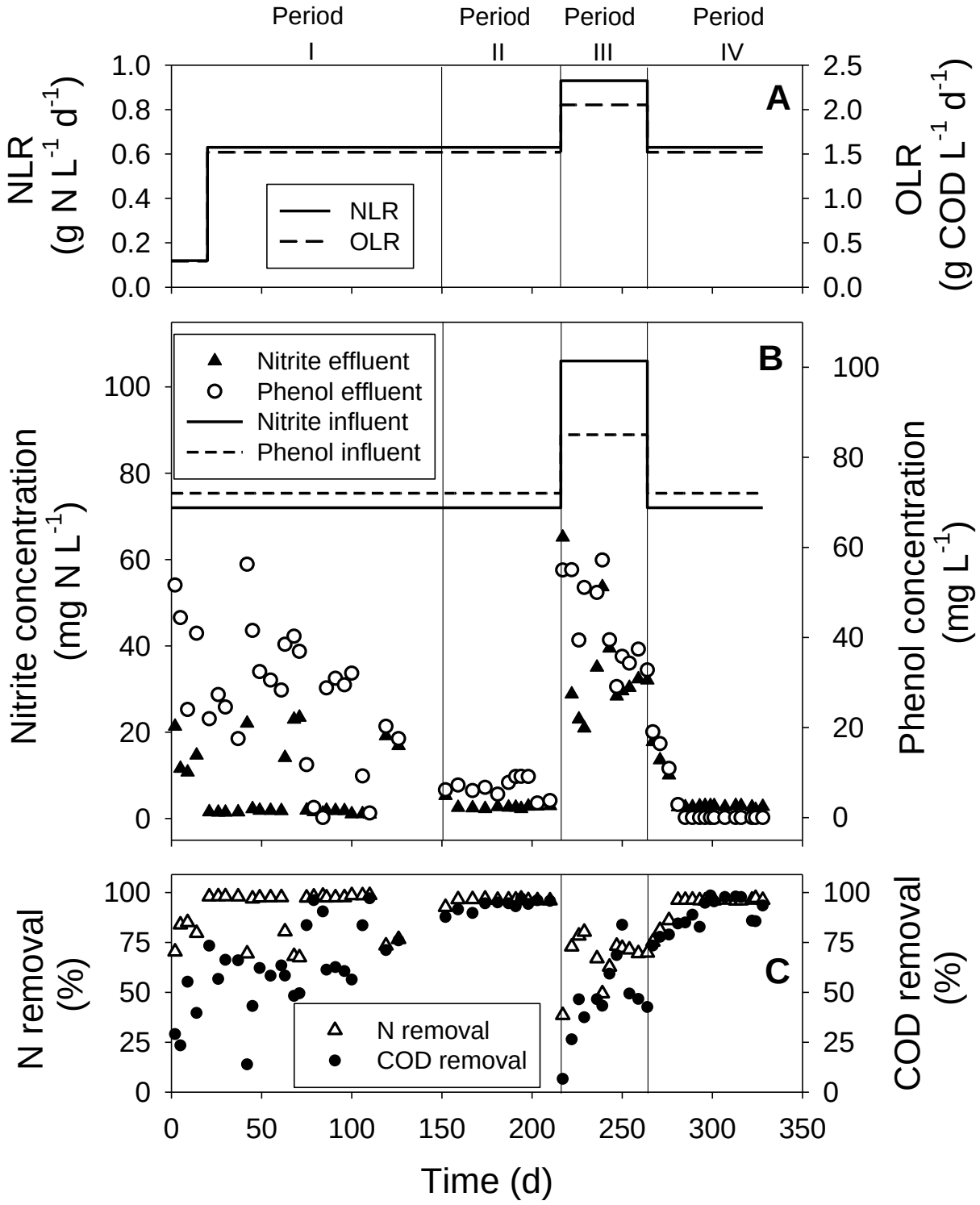

Figure 1

Figure

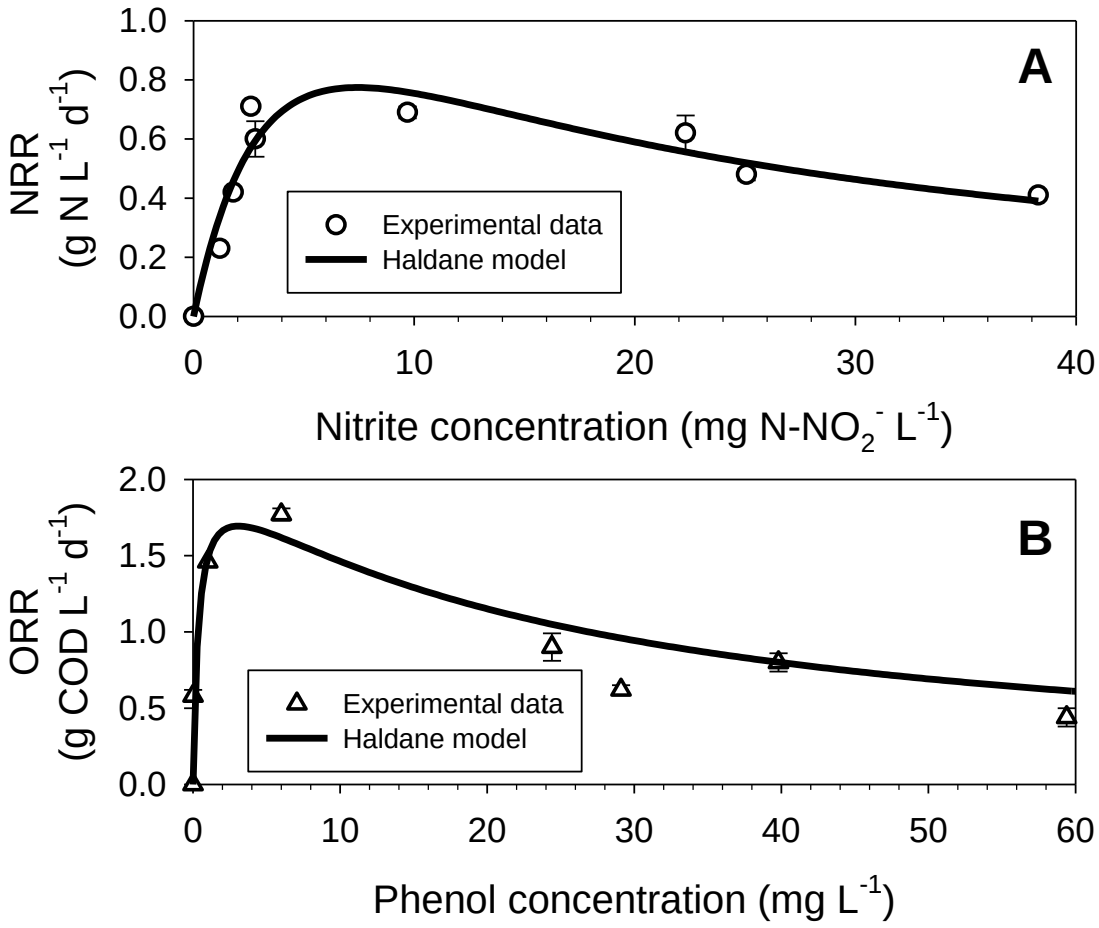

Figure 2

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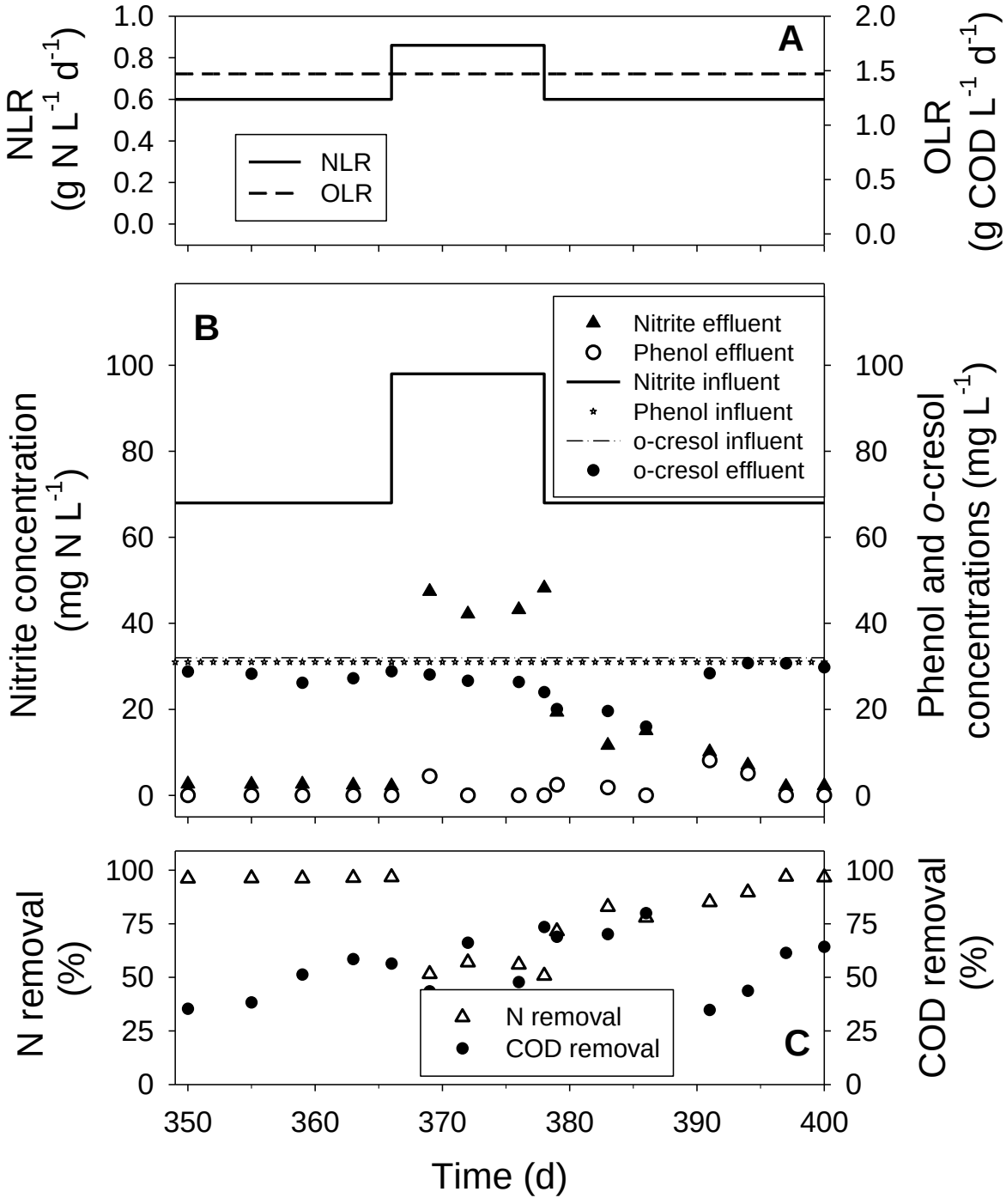

Figure 3

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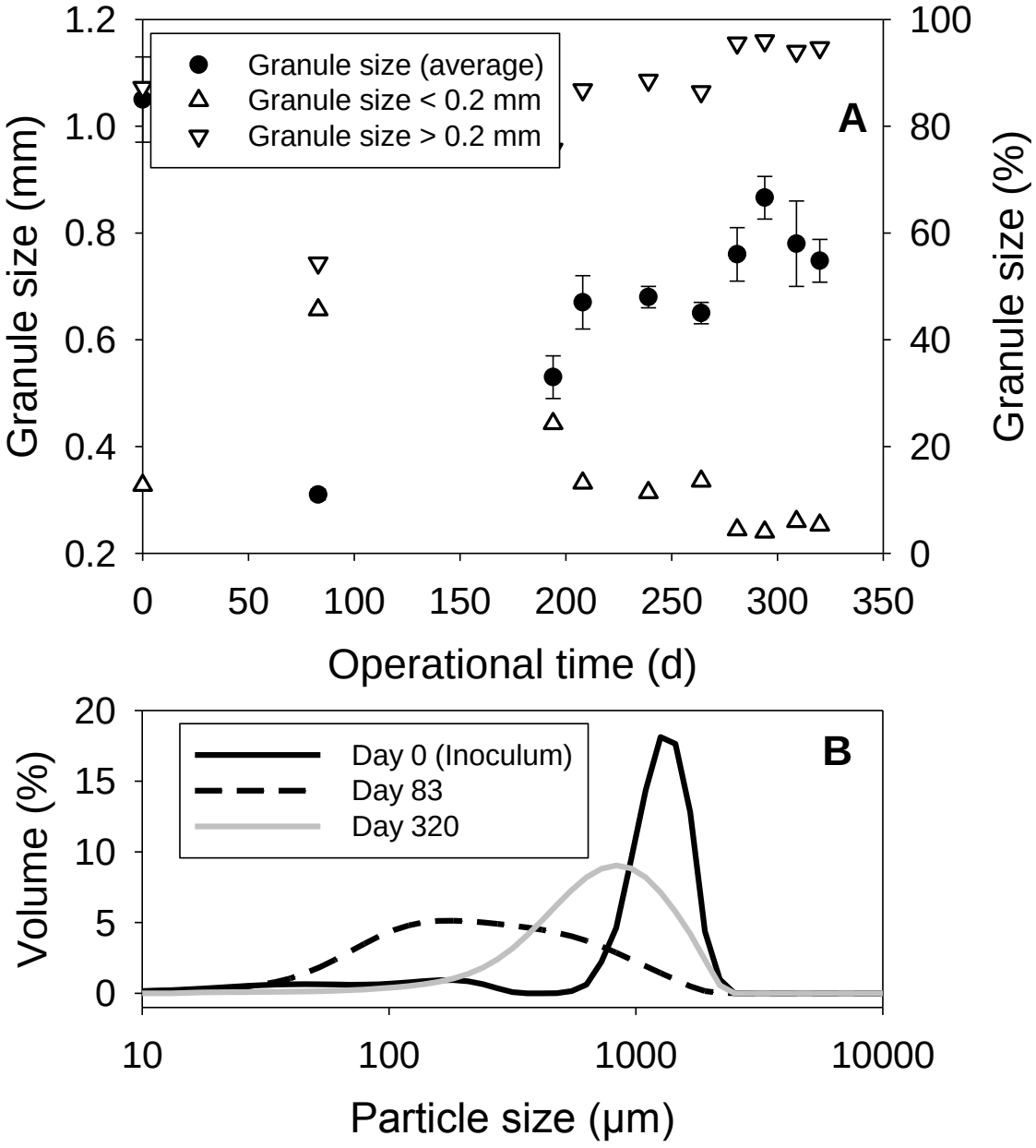

Figure 4

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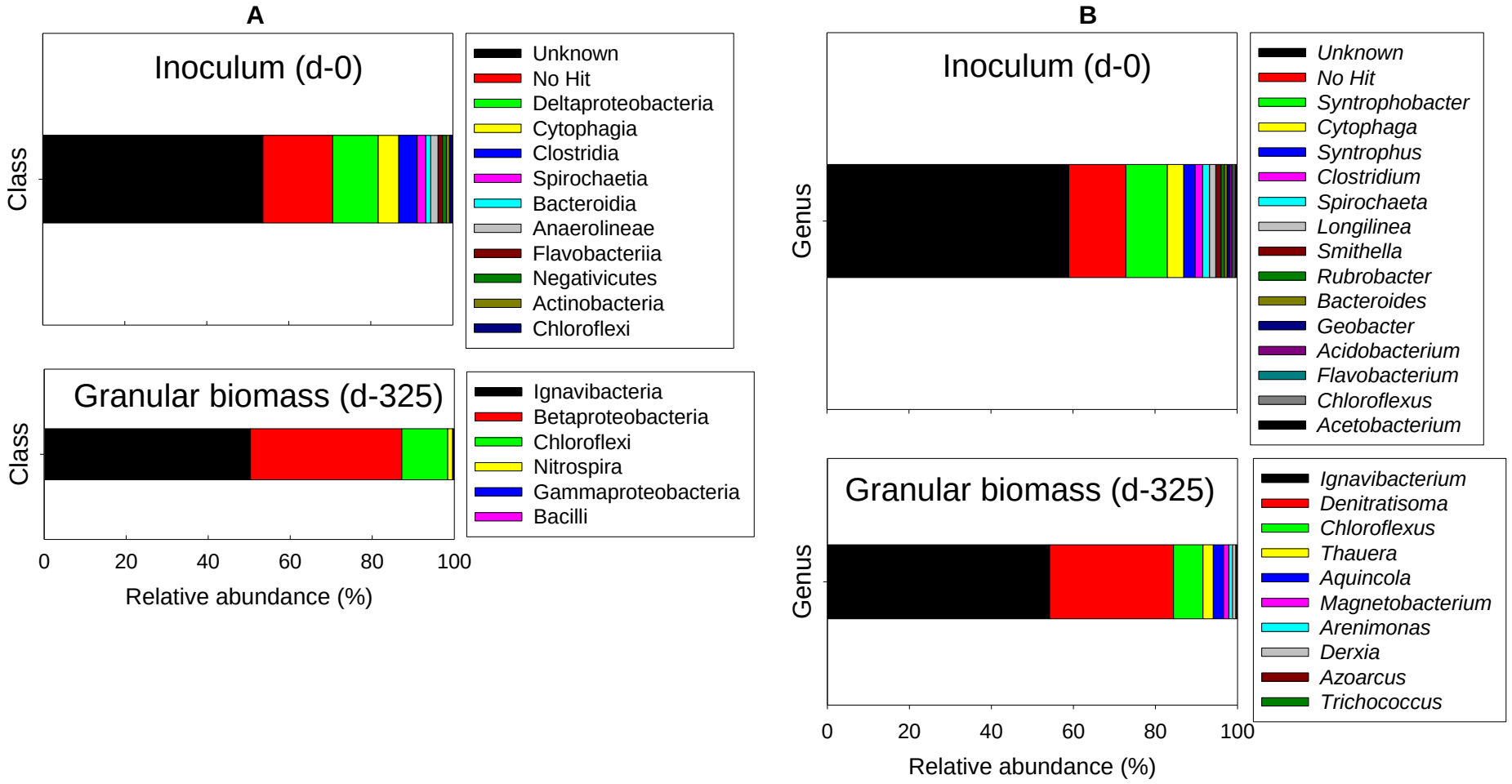

Figure 5