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Kinetic and microbiological characterization of aerobic granules performing partial nitritation of a low-strength wastewater at 10ºC

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Abstract

A granular airlift reactor enriched in ammonia oxidizing bacteria (AOB) was operated at 10 ºC performing stable partial nitritation in the long-term. The reactor treated a synthetic low-strength influent during 250 days with an average nitrogen loading rate of 0.63±0.06 g N L^{-1} d^{-1}. Nitrate production was barely detected, being the average concentration in the effluent of 0.6±0.3 mg N-NO_3 L^{-1}. Furthermore, a suitable effluent for a subsequent reactor performing the anammox process was achieved. A maximum specific growth rate as high as 0.63±0.05 d^{-1} was determined by performing kinetic experiments with the granular sludge in a chemostat and fitting the results to the Monod model. Pyrosequencing analysis showed a high enrichment in AOB (41 and 65 % of the population were identified as *Nitrosomonas* genus on day 98 and 233, respectively) and an effective repression of nitrite oxidizing bacteria in the long-term. Pyrosequencing

analysis also identified the coexistence of nitrifying bacteria and heterotrophic psychrotolerant microorganisms in the granular sludge. Some psychrotolerant microorganisms are producers of cryoprotective extracellular polymeric substances that could explain the better survival of the whole consortia at cold temperatures.

Keywords

Partial nitritation; mainstream; low temperature; NOB-repression; AOB-enrichment; anammox

1. Introduction

Nitrogen removal is essential in urban wastewater treatment plants (WWTPs) since nitrogenous compounds are toxic to aquatic life and cause eutrophication and oxygen depletion in receiving waters. Conventional activated sludge systems are the most frequently used systems in urban WWTPs since a good removal of pollutants is guaranteed, however the costs associated to this typical biological treatment make WWTPs as very energy-demanding facilities. For the achievement of a cost-effective (energy-neutral or even energy-positive) urban WWTP, the implementation of the autotrophic biological nitrogen removal (BNR) in the mainstream has been proposed (Jetten et al., 1997; Kartal et al., 2010; Siegrist et al., 2008). Thus, aeration costs are reduced because of the lower oxygen requirements of the process compared to conventional activated sludge treatment; and furthermore, biogas production is increased since most of the organic matter will be converted to biogas in the anaerobic digestion process, with the consequent energy recovery.

Recently, many studies were focused on the implementation of autotrophic BNR in one-stage systems, such as CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification/Denitrification) technologies. Nevertheless, at low temperature and low-strength wastewaters most of these systems showed the failure of nitritation in the long-term operation, due to the growth of nitrite oxidizing bacteria (NOB) triggering the production of nitrate and the destabilization of the subsequent anammox process (De Clippeleir et al., 2013; Hu et al., 2013; Wett et al., 2013; Winkler et al., 2011). Even though Gilbert et al. (2014) reported stable operation at 10 ºC with synthetic low-strength wastewater in a one-stage system, the achieved ammonium conversion rate resulted as low as 0.015 g N L^{-1} d^{-1}. Hence, two-stage systems appear as the alternative to overcome the destabilization problems and the low conversion rates associated to one-stage systems (Ma et al., 2011; Pérez et al., 2015; Regmi et al., 2014). Separation of the partial nitritation and the anammox process in two different reactors makes possible a more stable performance and control. In fact, stable partial nitritation at 12.5 ºC with a granular sludge reactor was reported by Isanta et al. (2015) and long-term operation of an anammox reactor at temperatures between 20 and 10 ºC was reported by Lotti et al. (2014). Both of these studies treated low-strength wastewater, demonstrating the feasibility of application of autotrophic BNR to mainstream conditions.

For both, one and two-stage approaches, successful implementation of autotrophic BNR at mainstream conditions relies on the stability of partial nitritation in the long-term, i.e. by achieving an effective repression of NOB activity. Previous research has shown a more sensitive temperature dependence of ammonia oxidizing bacteria (AOB) compared to that of NOB (Hunik et al., 1994; Knowles et al., 1965; Van Hulle et al., 2010). Thus, different strategies have been conducted in order to favour AOB over NOB activity at mainstream conditions. On one hand, Gao et al. (2014) proposed an aeration control strategy depending on the temperature and ammonia concentration in the influent. Efficient NOB repression was obtained at room temperature (12-27 ºC) but temperature fluctuated daily, being lower than 15 ºC less than 10 days. On the other hand, Isanta et al. (2015) achieved stable partial nitritation for 300 days at 12.5 ºC in a granular sludge system, by maintaining an adequate ratio between oxygen and ammonium concentrations in the reactor bulk liquid. However, in northern climates, temperature can easily achieve values lower than 12.5 ºC during winter. In fact, average temperatures of wastewater in west European region are around 17 ºC, with a minimum of 8 ºC and a maximum of 29 ºC (De Clippeleir et al., 2013), and thus, a temperature gradient from 20 ºC in summer to 10 ºC in winter was presented as representative for WWTPs in moderate climates (Gilbert et al., 2015).

In the present study we would like to demonstrate the long-term stability of partial nitritation at 10 ºC for low-strength synthetic wastewater in a granular sludge reactor operated in continuous mode. Furthermore, we aim for a better understanding of the process through the in depth study of the nitrifying biomass of the granular sludge reactor. Thus, our second objective is to characterize the population developed at low temperature in the reactor from both microbiological and kinetic points of view. Finally, we correlated the special characteristics of the biomass with the nitrifying ability of the granular reactor at 10 ºC.

2. Materials and methods

2.1. Reactor set-up and operation

A lab-scale airlift reactor with a total volume of 5.2 L, with a downcomer-to-separator diameter ratio of 0.36 and a total length-to-downcomer diameter ratio of 16 was used. The detailed diagram of the reactor is presented in Fig. 1. Compressed air was supplied through an air diffuser placed at the bottom of the reactor and was manually manipulated to maintain the dissolved oxygen (DO) concentration in the bulk liquid in the range 0.5-2.5 mg O_2 L^{-1}. The DO concentration in the bulk liquid was measured online by means of a DO electrode (DO 60-50, Crison Instruments, Spain). The pH was measured online with a pH probe (pH 52-10, Crison Instruments, Spain) and automatically controlled at 8.0±0.1 by dosing a Na_2CO_3 0.5 M solution. The pH was controlled throughout the operation period to rule out any potential effects derived from pH changes. Since the effect of pH on nitritation rates is known to be reduced in the range 7.5-8, a pH set point of 8 was selected, as done in a previous study (Isanta et al., 2015). The temperature was measured and controlled at 10 ºC by means of a cooling system (E100, LAUDA, Germany) and an electric heater (HBSI 0.8 m, HORST, Germany) connected to a temperature controller (BS-2400, Desin Instruments, Spain). Total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$) and nitrate concentrations in the bulk liquid were measured by using an on-line probe (AN-ISE sc probe with a Cartrical cartridge plus, Hach Lange, Germany). The range of the on-line probe for TAN and nitrate concentrations was 0-1000 mg N L^{-1} whereas the detection limit was 0.2 mg N L^{-1} for both parameters. TAN concentration in the bulk liquid was automatically controlled by varying the inflow rate by means of a proportional controller during the whole period of operation, except between days 93-95, 144-172 and 241-245 when the control was manually made based on the off-line bulk liquid TAN concentration measurement.

2.2. Wastewater and inoculum characteristics

The reactor treated a synthetic influent with an average TAN concentration of 70 mg N L^{-1}, which mimics a pretreated municipal wastewater coming from the mixture of the effluent of a previous A-stage plus the recirculation of the reject water of the digested sludge, as in an anammox-based WWTP (Isanta et al., 2015; Kartal et al., 2010). The synthetic influent also contained: 45 mg L^{-1} KH_2PO_4, 784 mg L^{-1} $NaHCO_3$, 80 mg L^{-1} NaCl, 40 mg L^{-1} $CaCl_2$, 90 mg L^{-1} $MgCl_2$ and 1 mL of trace elements solution per L of influent (Guerrero et al., 2011).

The biomass was enriched in AOB and adapted to low temperature (12.5 ºC) in a reactor which was operating for more than 400 days performing stable partial nitritation (Isanta et al., 2015). Hence, the inoculum contained around 81±12 % of AOB and 1±1 % of NOB as analyzed by fluorescent *in-situ* hibridization (FISH).

2.3. Kinetic experiments

Kinetic experiments were conducted in a chemostat with a working volume of 2.9 L. For each experiment, the chemostat was inoculated with nitrifying granules from the continuous airlift reactor to a final concentration of 83±3 mg VSS L^{-1}. The same synthetic wastewater of the reactor was used as influent to carry out the kinetic experiments. DO was measured in the bulk liquid and it was maintained in excess to avoid oxygen limitations (around 9 mg O_2 L^{-1}). Biomass was mixed both by mechanical stirring at 100 rpm (Stirrer type BS, VELP Scientifica, Italy) and bubbling of air to avoid mass transfer limitations. The pH was monitored and controlled at 7.5 by using an

ON/OFF control system by automated addition of 1 M NaOH with an automatic dispensing burette (Multi-Burette 2S-D, Crison Instruments, Spain). Temperature was maintained at 10 ºC by means of a cooling system (E100, LAUDA, Germany), which provided cooled water through the jacket of the chemostat. The measurement of the particle size of the biomass of both the effluent and the reactor confirmed that biomass was not retained in the reactor and consequently the operation was as a chemostat (Fig SI-1 in Supporting Information).

Taking into account that the dilution rate is equal to the growth rate (μ) in a chemostat, growth rate was fixed by varying the dilution rate, and thus the inflow. The chemostat was operated continuously and experiments were finished when steady state conditions were achieved, that is, when TAN concentration in the effluent was constant. Five experiments were carried out at different growth rates ranging from 0.36 to 0.56 d^{-1}, and a value of TAN concentration at steady state conditions was obtained for each experiment.

Kinetic parameters (maximum specific growth rate, μ_{max}, and TAN affinity constant, $K_{S,TAN}$) were determined by fitting the data of the experiments to the Monod equation (Eq. 1).

$$\mu = \mu_{max} \frac{[TAN]}{K_{S,TAN} + [TAN]} \qquad \text{(Eq. 1)}$$

2.4. Analytical methods

Total ammonia nitrogen concentration was measured off-line with an ammonium analyzer (AMTAX sc, Hach Lange, Germany), total nitrite nitrogen (TNN = N-NO_2^- +

N-HNO_2) and nitrate concentrations were analyzed off-line with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). These measured off-line values are the ones represented in the results section. Mixed liquor total suspended solids (TSS) and mixed liquor volatile suspended solids (VSS) were analyzed according to Standard Methods (APHA, 1999). SRT was estimated by dividing the amount of VSS in the reactor by the sludge washed out with the effluent:

$$SRT = \frac{[VSS]_{reactor} * V_{reactor}}{[VSS]_{effluent} * Q_{effluent}} \qquad (1)$$

Where, $[VSS]_{reactor}$ and $[VSS]_{effluent}$ are the VSS concentrations in the reactor and the effluent, respectively, $V_{reactor}$ is the reactor volume and $Q_{effluent}$ is the effluent flow rate.

Average particle size was measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). The off-gas of the reactor was periodically collected and analyzed with gas chromatography (Agilent Technologies 6890 N Network GC system, Madrid, Spain) to measure N_2O emissions.

2.5. Fluorescence in situ hybridization (FISH) analysis

Abundances of AOB and NOB were analyzed by FISH coupled to confocal laser scanning microscopy (CLSM). Regarding AOB, specific probes for *Nitrosomonas* spp. and *Nitrosospira* spp. were 5’-6FAM-labeled and 5’-Atto550-labeled, respectively. Regarding NOB, specific probes for *Nitrobacter* spp. and *Nitrospira* spp. were 5’-Cy3-labeled and 5’-6FAM-labeled, respectively. Hybridizations were performed with the specific and general (5’-Cy5-labeled) probes described in Table SI-1 in Supporting Information. Biomass samples were grabbed from the reactor and granules were crushed by means of a mortar and a pestle in order to ease hybridization. A Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) using a Plan-Apochromatic 63x objective (NA 1.4, oil) was used to quantify biomass by analyzing 30-40 fields and following an automated image analysis procedure as described in Jubany et al. (2009).

2.6. Pyrosequencing analysis

Identification of the microbial population was performed using next-generation sequencing at day 98 and 233 of the reactor operation. DNA was extracted from biomass samples by applying the protocol of MoBio PowerBiofilm™ DNA extraction kit (MoBio Laboratories, USA). Two modifications of the manufacturer protocol were performed: 200 mL of solution BF3 were added instead of the 100 mL recommended, and 80 mL of solution BF7, instead of the 100 mL recommended. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the quantity and quality of extracted DNA. A minimum of 20 ng L^{-1} of extracted DNA was guaranteed to perform 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 V2-V4 were targeted using the primer pair 341F-907R. More information about the bioinformatics applied to the sample can be found in Supporting Information.

2.7. Scanning electron microscopy

A biomass sample of 8-10 granules was fixed in 2.5 % (v/v) glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C, washed 4 times for 10 min each time in 0.1 M phosphate buffer, fixed in 1 % (wt/v) osmium tetraoxide with 0.7 % ferrocyanide in phosphate buffer, washed in water, dehydrated in an ascending ethanol series (50, 70, 80, 90, and 95 % for 10 min each and twice with 100 % ethanol), and dried at critical-point with CO_2. Then, the sample was metalized with Au-Pd and observed by using a scanning electron microscope (EVO MA10; Zeiss, Germany) at the following conditions: 20 kV, 100 pA, secondary electron detector (SE1).

3. Results and discussion

3.1. Long-term operation at 10 ºC

The reactor was previously operated at 12.5 ºC, with an average NLR of 0.7±0.3 g N L^{-1} d^{-1}, for more than 400 days performing stable partial nitritation before the temperature was directly lowered to 10 ºC (Isanta et al., 2015). After the decrease in temperature (day 0), the reactor was operated during 250 days with an average nitrogen loading rate (NLR) of 0.63±0.06 g N L^{-1} d^{-1}. Stable partial nitritation was maintained in the long-term at 10 ºC (Fig. 2), which was achieved by applying a ratio of DO/TAN concentrations in the bulk liquid of 0.04±0.02 mg O_2 mg^{-1} N. Low DO/TAN concentrations ratio was reported before to maintain stable partial nitritation in granular systems (Bartrolí et al., 2010; Isanta et al., 2015; Jemaat et al., 2013). Efficiency of the NOB repression is thought to be linked to the fact that a steep oxygen gradient is present in the granular sludge (Bartrolí et al., 2010; Isanta et al., 2015). Therefore, direct extrapolation of this strategy to other systems, such as flocculent sludge reactors in which sludge retention is assured by other means, it is not straightforward but might be object of future research. Nitrate production was barely detected, being the average concentration in the effluent of 0.6±0.3 mg N-NO_3^- L^{-1}. Furthermore, a suitable effluent for a subsequent reactor performing the anammox process was achieved, with an average TNN/TAN concentrations ratio of 1.1±0.2. The ammonium oxidation rate

(AOR) was maintained stable during the whole operation, with an average value of 0.34±0.06 g N L^{-1} d^{-1}. This value is considerably high compared to the one obtained in one-stage biofilm systems. Thus, Gilbert et al. (2015) reported an AOR lower than 0.02 g N L^{-1} d^{-1} at 10 ºC and Hu et al. (2013) reported an AOR of 0.03 g N L^{-1} d^{-1} at 12 ºC.

Particle size was maintained stable during the whole period of operation (Fig. 2A) with an average value of 810±70 µm. From day 50 onwards, the biomass concentration increased to an average value of 3.6±0.1 g VSS L^{-1}, as shown in Fig. 2A. In spite of the high and constant NLR and AOR achieved in the granular airlift reactor, specific rates (specific nitrogen loading rate, sNLR; specific ammonium oxidation rate, sAOR) decreased during the first 100 days at 10 °C (Fig. 2C). However, sAOR remained constant from day 100 with an average value of 0.18±0.03 g N mg^{-1} VSS d^{-1}. This fact demonstrated that biomass maintained the same activity during 150 days of operation at 10 ºC.

Between days 93-95, 144-172 and 241-245 the ammonium control was switched from automatic to manual, but the process remained stable. Moreover, on day 98 the reactor remained without feeding for 4 hours (NLR of 0 g N L^{-1} d^{-1}) which resulted in an increase of DO and complete oxidation of ammonium to nitrite by AOB. Despite of the high concentration of TNN and DO, the nitrate in the bulk liquid was only 2.2 mg L^{-1} and the next day the system was totally recovered. Hence, the successfully repression of NOB in the system was demonstrated and thus, the stability of this technology.

The balance of nitrogen during the operation of the reactor was fulfilled, with an average value of 96±6 % (Figure SI-2 in Supporting Information). Hence, neither heterotrophic nor autotrophic (anammox process) denitrification was considered to take place in the granular airlift reactor.

Off-gas samples from days 94, 95, 241, 242 and 245 were analyzed in order to calculate the N_2O emission factor of the reactor. As it is shown in Table 1, less than 0.35 % of the TAN in the influent was emitted as N-N_2O. Furthermore, from the converted nitrogen the average emitted as N-N_2O was 0.36±0.07 %. Thus, the N_2O emissions from the granular airlift reactor performing partial nitritation of a low-strength wastewater at 10 °C were very low, even at low DO concentrations (1.3±0.3 mg O_2 L^{-1}) which is known to trigger high N_2O emissions (Kampschreur et al., 2009). Applying the same control strategy but treating a reject water from the dewatering of digested sludge (high-strength wastewater) at 30ºC, the N_2O emission factor was as high as 6 % of the TAN oxidized at a DO of 1 mg O_2 L^{-1} (Pijuan et al., 2014), which means more than one order of magnitude higher than the reported in this study. Hence, the temperature could be an important factor affecting N_2O emissions, the lower the temperature the lower the emissions. Nevertheless further experiments are necessary to confirm this hypothesis, since other factors could cause the difference between the results of Pijuan et al. (2014) and this study.

3.2. Kinetics

As it was mentioned before, the granular airlift reactor not only achieved stable partial nitritation at 10 ºC but also operated at higher NLR than other similar systems. Nitrifying capacity is related to the growth rate of AOB community and, hence, a nitrifying sludge with an unusually high maximum growth rate could explain the high activity in this airlift reactor.

The results of the five kinetic experiments carried out in a chemostat reactor are presented in Table 2. An increase in the applied growth rate caused an increase in the TAN concentration at steady state conditions, which follows satisfactorily (R^2 = 0.97) the Monod kinetic model (Figure 3). Hence, the maximum specific growth rate and TAN affinity constant were obtained by fitting the data achieved in each experiment to the Monod kinetic model. Thus μ_{max} and $K_{S,TAN}$ resulted in 0.63±0.05 d^{-1} and 2.1±0.7 mg N L^{-1}, respectively.

Different values of maximum growth rate have been reported up to now, being most of them determined at high temperatures (20-30 ºC) (Blackburne et al., 2007; Esquivel-Rios et al., 2014; Vadivelu et al., 2006). However, large discrepancies were found between different studies. The large variety of parameter values found in literature lies in the differences of systems evaluated, operational conditions applied, biomass growth types and the techniques used to determine the parameters themselves. Vannecke and Volcke (2015) presented a literature review on microbial characteristics of nitrifiers and reported μ_{max} in the range of 0.34-3.40 d^{-1} for attached growth of AOB at 30 ºC and pH 7.5. For suspended growth, Farges et al. (2012) used the flow cytometry technique to study the growth of *Nitrosomonas europaea* in pure cultures at 26 ºC and pH 8 and μ_{max} resulted in the range of 0.13-0.23 d^{-1}. On the other hand, Chandran et al. (2008) obtained higher values (μ_{max} = 0.24-0.74 d^{-1}) by using respirometric batch tests and substrate depletion assays in continuous reactors for an enriched nitrifying culture at 25 ºC and pH 7.4.

It is well known that growth rate decreases considerably with decreasing temperature. Knowles et al. (1965) reported a decrease in the μ_{max} from 1.5 to 0.2 d^{-1} when temperature decreased from 27 to 8.3 ºC for *Nitrosomonas* sp. in samples from Thames estuary, London, England; and afterwards, Sözen et al. (1996) determined μ_{max} in the range of 0.10-0.17 d^{-1} for a nitrifying mixed culture treating real urban wastewater at 10 ºC. In spite of this, little has been published about kinetic parameters of nitrifying mixed cultures at low temperature, and in any case, the μ_{max} values reported were much lower than the one achieved in the current study ($\mu_{max} = 0.63 \pm 0.05$ d^{-1} at 10 ºC). In fact, to the best of the authors' knowledge, this is the highest growth rate achieved by a nitrifying sludge enriched in AOB at 10 ºC. Furthermore, an estimation of the μ_{max} of the nitrifying sludge at higher temperatures was calculated by considering an Arrhenius-type equation ($\mu_{1,T_1} = \mu_{2,T_2} \cdot \theta^{(T_1-T_2)}$) and a temperature coefficient of $\theta = 1.13 \pm 0.03$ which was determined in Isanta et al. (2015) for the inoculum of the current airlift reactor. The values obtained for μ_{max} were 2.1±0.2, 3.9±0.3 and 7.3±0.6 d^{-1} at 20, 25 and 30 ºC, respectively. Thus, the nitrifying biomass of the granular airlift reactor presented the higher μ_{max} than has been reported hitherto at any temperature. A nitrifier culture with such a high μ_{max} could explain the high NLR and AOR achieved in the operation of the granular airlift reactor at 10 ºC. A second implication is that the enrichment of an AOB population with such a high μ_{max} would be an advantage for NOB repression at low temperatures, since it would help to keep AOB growth rate higher than that of NOB.

Along with the high μ_{max} obtained, a high value for the TAN affinity constant was determined from the kinetic experiments ($K_{S,TAN} = 2.1 \pm 0.7$ mg N L^{-1}) compared to the previously reported by Chandran et al. (2008) at 25 ºC ($K_{S,TAN} = 0.21$-0.69 mg N L^{-1}), Knowles et al. (1965) at 8.3 ºC ($K_{S,TAN}$ = 0.2 mg N L^{-1}) and the proposed in the Activated Sludge Model 2d at 10 ºC ($K_{S,TAN}$ = 1 mg N L^{-1}; Henze et al, (2000)).

From the ecological concept, a microorganism showing quick growth on easily available substrate is defined as r-strategist microorganism (Andrews and Harris, 1986), which applied to the kinetic context represents a microorganism with high maximum specific growth rate and high substrate affinity constant (Andrews and Harris, 1986; Martín-Hernández et al., 2009). Therefore, the nitrifier population of the granular airlift reactor can be considered as r-strategist. The enrichment of a r-strategist AOB population has been reported when high residual ammonium concentrations are used (Terada et al., 2013). Hence, the high residual ammonium concentration may be also a key factor for the enrichment of r-strategist AOB population in two-stage partial nitritation/anammox reactor systems, like the one presented in this study.

3.3. Microbial characterization

FISH-CSLM was used to evaluate the enrichment in AOB and the presence of NOB in the granular sludge performing partial nitritation at 10 ºC. On day 233, 92±4% of the population was quantified as AOB and less than 1±1% as NOB (specifically *Nitrobacter* spp*)*. Since the inoculum contained 81±12% of AOB and 1±1% of *Nitrobacter* spp., a high enrichment in AOB and an effective repression of NOB was maintained in the long-term at 10 ºC although NOB were always present in the biomass.

On the other hand, neither *Nitrosospira* spp. (species belonging to AOB) nor *Nitrospira* spp. (species belonging to NOB) hybridizations were detected in the sludge. This fact was expected since they are k-strategist microorganisms and, consequently, they are not favored at high TAN and TNN concentrations (Kim and Kim, 2006), such as those in the reactor of this study.

Moreover, pyrosequencing technique was used to examine the microbial community through the operation of the granular reactor at 10 ºC. With that purpose, samples on days 98 and 233 were analyzed.

On sample from day 98, *Betaproteobacteria* was clearly the most abundant class of the total reads, with a relative abundance of 52 % (Fig 4). It is widely known that *Betaproteobacteria* class comprises autotrophic nitrifying microorganisms, such as AOB and NOB, and also denitrifying bacteria and organic matter decomposing bacteria. Thus, it is expected that *Betaproteobacteria* was the most abundant class in an AOB enriched sludge, such as the one of this study. *Alphaproteobacteria* was the second class in order of abundance, with a value of 23 %, following by *Actinobacteria* and *Gammaproteobacteria* representing the 7 and 5 % of total reads, respectively, among other classes of heterotrophs less abundant in the sample. These values of heterotrophic classes are in the range of the observed by Kindaichi et al. (2004), with 23 % of *Alphaproteobacteria* and 13 % of *Gammaproteobacteria* quantified in an autotrophic nitrifying biofilm system operating at 25 ºC and with a high-strength synthetic wastewater.

On sample from day 233, corresponding to long-term operation of the granular reactor at 10 ºC, *Betaproteobacteria* increased their relative abundance to 68%, followed by the increasing of *Cytophagia* with a 15 %; while *Alphaproteobacteria* sharply decreased to 4 % (Fig. 4). There was a 10 % of reads not identified at class level, and most of them comprised the phylum *Bacteroidetes*. In fact, *Bacteroidetes* abundance increased significantly compared to day 98, being the phylum with the highest increase (Figure SI-3 in Supporting Information).

At genus level, in the biomass community of day 98 (Fig. 5), *Nitrosomonas* was the most abundant genus, which was expected since the sludge was enriched in AOB and *Nitrosomonas* is the most frequently genus of AOB found in wastewater treatment systems (Wagner et al., 2002; Wang et al., 2012). Thus, *Nitrosomonas* genus counted up the 41% of the total population, indicating a majority of AOB in the sludge. Since *Nitrosomonas* genus comprises r-strategist microorganisms (Terada et al., 2013), their high abundance in the nitrifying sludge agreed with the high values of μ_{max} and $K_{S,TAN}$ obtained from kinetic experiments. Besides, *Nitrosospira* genus was also detected in the sample with a 7 % of relative abundance, in spite of *Nitrosospira* spp. were never detected by FISH technique. This may be due to the fact that FISH technique points toward the abundance of rRNA in samples, while pyrosequencing points toward the abundance of DNA (Wittebolle et al., 2005). Thus, *Nitrosospira* spp. could be not detected by FISH because their probably low or null activity in the reactor, but their DNA could be still detected. Regarding NOB, 1.4 % of the total population was identified as *Nitrobacter* genus, which agrees with the production of nitrate in the reactor and with the result of the FISH analysis. Thus, *Nitrobacter* spp. were not abundant in the reactor, but active. Finally, *Nitrospira* genus was not detected in the sample, in agreement with the FISH analysis.

Other genera were detected in a low abundance, but in a more or less equal proportion between them. High bacteria richness is expected in mixed cultures and the coexistence and interaction of heterotrophic bacteria and autotrophic nitrifiers were reported before (Ducey et al., 2010; Kindaichi et al., 2004; Okabe et al., 2005). In this sense, genera as *Sphingomonas* and *Dokdonella*, with a relative abundance in the sample of 8 and 4 % respectively, were reported as heterotrophic, or even autotrophic nitrifiers (Fitzgerald et al., 2015). Moreover, two other genera, *Cryobacterium* and *Flavobacterium*, with several species known to be either psychrotolerant (*Cryobacterium psychrotolerans*, Zhang et al. (2007); *Flavobacterium gelidilacus*, Van Trappen et al. (2003)) or even psychrophilic (*Cryobacterium* sp. MLB-32, Singh et al. (2015)) microorganisms, were identified in the sample with 7 and 5 % of the total reads, respectively.

On day 233 at genus level (Fig 5), enrichment in *Nitrosomonas* genus was observed to the detriment of the rest of genera. Moreover, *Nitrosomonas* genus counted the 65 % of the total reads in the sludge sample, being at day 233 the 96 % of all the *Betaproteobacteria* while at day 98 this percentage was of 78 %. Thus, the sludge was much more enriched in AOB at day 233 than at the beginning of the operation at 10 ºC. *Nitrosospira* genus was present in the sample in less than 0.5 % of abundance, so it was not considered for the data treatment. Since *Nitrosospira* was found on day 98 (7 % of the reads), pyrosequencing analysis confirmed its wash-out from the granular airlift reactor operating at 10 ºC. Furthermore, the absence of *Nitrosospira* genus at day 233 confirms that *Nitrosospira* were not active on day 98 (no detected by FISH) and, hence, they were washed out of the reactor. Besides, since the reactor was operated at high solid retention time (80±20 days) the wash-out was slow. There may be two reasons for the wash-out of *Nitrosospira* genus in the granular airlift reactor. The first one is that *Nitrosospira* spp. were not favoured at the operating conditions of the reactor since they are k-strategist microorganisms (low TAN affinity constant and low specific growth rate) while *Nitrosomonas* spp. are r-strategists (high TAN affinity constant and high specific growth rate). The second possible explanation is that *Nitrosospira* spp. are more sensitive to temperature than *Nitrosomonas* spp (Hoang et al., 2014; Park et al., 2008).

Neither *Nitrobacter* nor *Nitrospira* genera were identified by pyrosequencing in the sample of day 233, in spite of being detected with the FISH analysis (with an abundance of 1±1 %). Probably this could be due to poor or null amplification of a low DNA content of these bacteria in the sample. In any case, successful NOB repression in the granular airlift reactor operating at 10 ºC was demonstrated.

As it is shown in Fig. 5, the second genus in order of abundance with a 15% of the total reads was unclassified at genus level (classified at order level as *Cytophagales*), however its DNA sequence was ran against BLAST and matched the one found by Larose et al. (2010) in bacteria present in snow and melt water samples from Svalbard, Norway. Therefore, the corresponding microorganism will probably be a cold-adapted microorganism (psychrotolerant or psycrhrophilic species) and its presence in cold waters would fit with the presence in the 10 ºC system of this study.

In general terms, in addition to the enrichment in AOB, three main points can be extracted from the results obtained by pyrosequencing in this study. The first one is that the diversity of the bacterial community decreased in the long-term of operation at 10 ºC. The second one is that despite the fact that the influent of the reactor was devoid of an organic carbon source, a considerable part of the population in both samples was composed by heterotrophic bacteria. Finally, the third one is the presence of psychrotolerant microorganisms in the sludge performing partial nitritation at 10 ºC.

As shown in Table 3, there were more genera with abundances higher than 5 % on day 98 than on day 233. The decrease in bacterial diversity with cold temperature was reported before (Karkman et al., 2011). Thus, not only non-adapted microorganisms to cold temperatures diminished in the long-term operation, but also diversity of psychrotolerant genera (the unclassified microorganism mentioned before appears to the detriment of *Cryobacterium* and *Flavobacterium*). Only *Nitrosomonas* and the unclassified genera (one of them corresponding to the cold-adapted microorganism mentioned before) were identified with abundance superior to 5 % on day 233.

The coexistence of nitrifying and heterotrophic bacteria in absence of organic carbon has also been reported before (Ducey et al., 2010; Hoang et al., 2014; Karkman et al., 2011). It is known that nitrifiers produce organic matter from biomass decay and substrate metabolism which is used by heterotrophs to survive. Nogueira et al. (2005) correlated the presence of heterotrophs in nitrifying biofilm reactors with the hydraulic retention time (HRT) and determined that values of HRT in the range of the one used in this study (2.5 ± 0.3 h) guarantees enough soluble microbial products (SMP) available for heterotrophic growth. There are also studies focused on the determination of these SMP derived from nitrifiers that can be used by heterotrophs (Kindaichi et al., 2004; Okabe et al., 2005).

Interaction between nitrifiers and heterotrophs is not only profitable for heterotrophic bacteria, but also for nitrifiers, becoming a synergic system as it was suggested by

Ducey et al. (2010) and Hoang et al. (2014). Some psychrotolerant and psychrophilic bacteria have been identified as producers of cryoprotective extracellular polymeric substances (EPS) that allow a better survival of the whole consortium at cold temperatures (Ducey et al., 2010). Therefore, this protection would affect in the same way to AOB, which could maintain the nitritation even when conditions were not ideal for their growth. Psychrotolerant microorganisms were found in both samples of the granular sludge and thus, they were present during the whole operation of the granular reactor at 10 ºC. Hence, although in our system the heterotrophic population was less significant than that of nitrifiers, its presence could be essential for the maintenance of the nitritation in the granular reactor. Fig. 6 shows a SEM image of the surface of a granule where bacteria seem to be embedded in a high amount of extracellular polymeric substances, which could be the cryoprotective EPS.

This study revealed high nitrifier ability for performing partial nitritation at 10 ºC: stable operation was maintained in the long-term in the granular airlift reactor and kinetic experiments showed the higher μ_{max} than has been hitherto determined for a nitrifying sludge at low temperature. We have two hypotheses to explain it: (i) AOB were cultivated in the long-term under low temperatures which could lead to a metabolic adjustment of the biomass and thus, to improve the ability to nitrify under this condition; (ii) granules comprised a consortium of microorganisms which included producers of cryoprotective EPS that give an adaptive advantage to AOB, protecting them from low temperatures.

4. Conclusions

Stable partial nitritation at 10 ºC was maintained in the long-term in a granular airlift reactor operating at high NLR.

The nitrifier culture enriched in AOB presented a significantly high μ_{max} compared to other studies which allowed the operation at high nitritation rates, being advantageous for NOB repression.

The microbial community was dominated by AOB (specifically *Nitrosomonas* genus) throughout the whole operation of the reactor; while NOB genera were barely detected, demonstrating their effective repression from the system. Effective NOB repression was not only achieved, but also it was obtained a suitable effluent for a subsequent reactor performing the anammox process.

The operation of the granular reactor in the long-term at 10 ºC with a high residual ammonium concentration decreased the microbial diversity and further enriched the granular sludge in AOB.

Partial nitritation at 10 ºC can be operated with low N_2O emissions since less than 0.35 % of the TAN in the influent was emitted as N-N_2O.

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Figure captions and table legends

Fig. 1 Schematic diagram of the reactor set-up showing the peripheral instrumentation and control loops. DO: dissolved oxygen; TAN: total ammonia nitrogen (TAN = N-NH_4^+ + N-NH_3).

Fig. 2 Continuous operation of the granular airlift reactor treating a synthetic low-strength wastewater at 10 °C. (A) Biomass concentration and particle size; (B) Nitrogen loading rate (NLR), and ammonium oxidation rate (AOR); (C) Specific nitrogen loading rate (sNLR), specific ammonium oxidation rate (sAOR) and dissolved oxygen concentration (DO); (D) Nitrogen compounds concentrations throughout the operation of the granular reactor.

Fig. 3 TAN oxidation kinetics for the nitrifying granules. (○) TAN concentration at steady state conditions for each specific growth rate imposed.

Fig. 4. Microbial diversity at class level. Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 0.5 % of the total copies.

Fig. 5. Microbial diversity at genus level. Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 0.5 % of the total copies.

Fig. 6 Scanning electron microscopy (SEM) images of a granule surface. Granule sample was taken on day 45 of operation at 10 °C. (A) 1,000x magnification; (B) 10,000x magnification; (C) 20,000x magnification.

Table 1 – N_2O emission factors during the operation of the granular airlift reactor treating a synthetic low-strength wastewater at 10 °C.

Table 2 – Operational parameters of the kinetic experiments in the chemostat at steady state conditions. All the experiments were conducted with the same influent of the main reactor at pH = 7.5±0.1, DO = 9.3±0.1 mg O_2 L^{-1} and T = 10 °C.

Table 3 – Genera with relative abundance higher than 5 % in samples of sludge on day 98 and 233.

Table 1

Day	$N\text{-}N_2O$ (% of N-influent)	$N\text{-}N_2O$ (% of N-oxidized)
94	0.13±0.01	0.23±0.02
95	0.32±0.04	0.58±0.08
241	0.23±0.04	0.40±0.08
242	0.17±0.06	0.30±0.10
245	0.14±0.02	0.28±0.04

Table 2

Number of Experiment	Experiment duration (days)	μ (d^{-1})	Inflow ($L\ d^{-1}$)	$[TAN]_{effuent}$ ($mg\ N\ L^{-1}$)	$[TNN]_{effluent}$ ($mg\ N\ L^{-1}$)	$[N\text{-}NO_3^-]_{effluent}$ ($mg\ N\ L^{-1}$)
1	13	0.55	1.60 ± 0.01	26.3 ± 0.7	49.0 ± 2.0	1.9 ± 0.1
2	10	0.34	1.00 ± 0.01	3.4 ± 0.2	30.0± 4.0	36.0 ± 1.0
3	13	0.53	1.53 ± 0.01	10.2 ± 0.6	47.0 ± 2.0	10.8 ± 0.4
4	9	0.56	1.62 ± 0.01	10.0 ± 1.0	56.0 ± 2.0	12.0 ± 1.0
5	16	0.45	1.30 ± 0.01	4.3 ± 0.4	34.0 ± 9.0	31.0 ± 7.0

Table 3

Day 98		Day 233	
Nitrosomonas	(41%)	*Nitrosomonas*	(65%)
Nitrosospira	(7%)	Unclassified (*Cytophagales* Order)	(15%)
Sphingomonas	(8%)	Unclassified (*Bacteroidetes Phylum*)	(8%)
Sphingopyxis	(5%)		
Cryobacterium	(7%)		
Flavobacterium	(5%)		
Comamonas	(5%)		

Figure

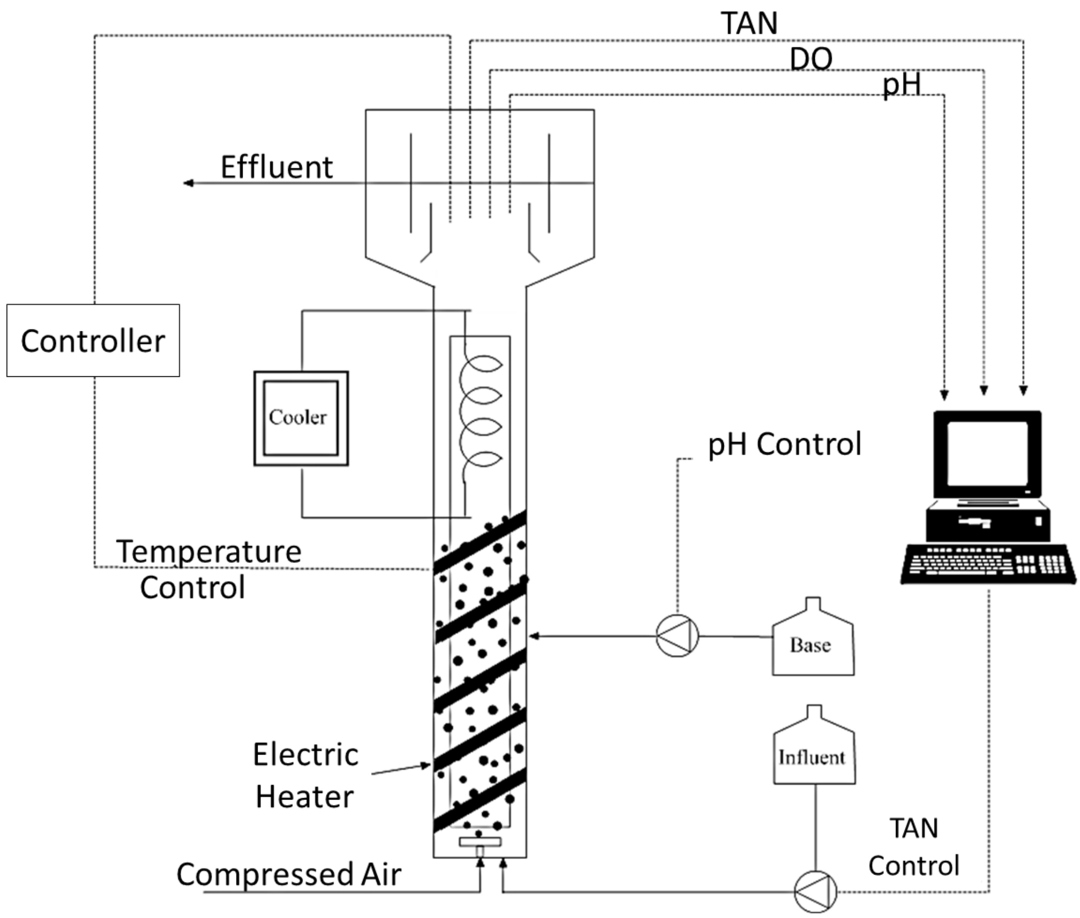

Figure 1

Figure

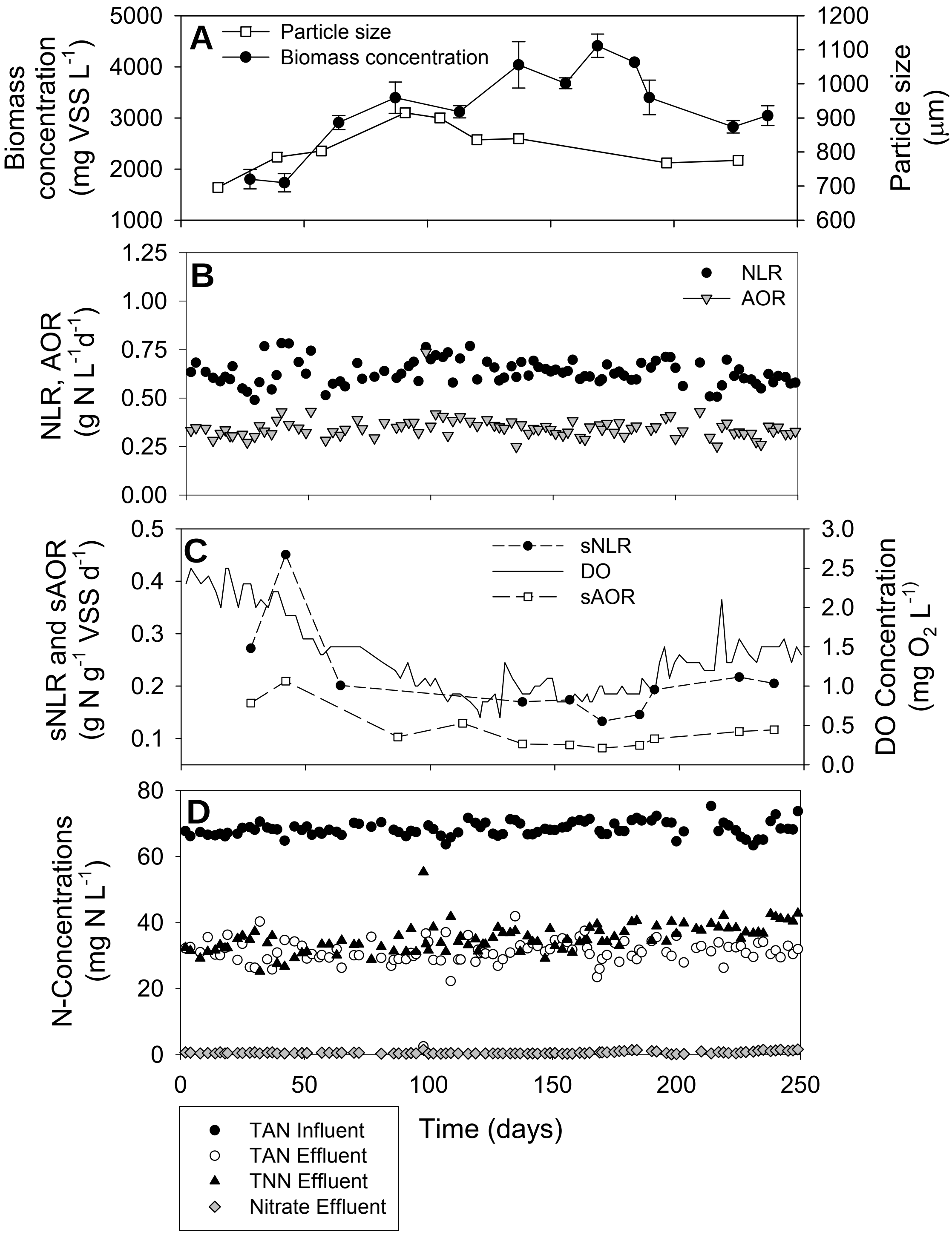

Figure 2

Figure

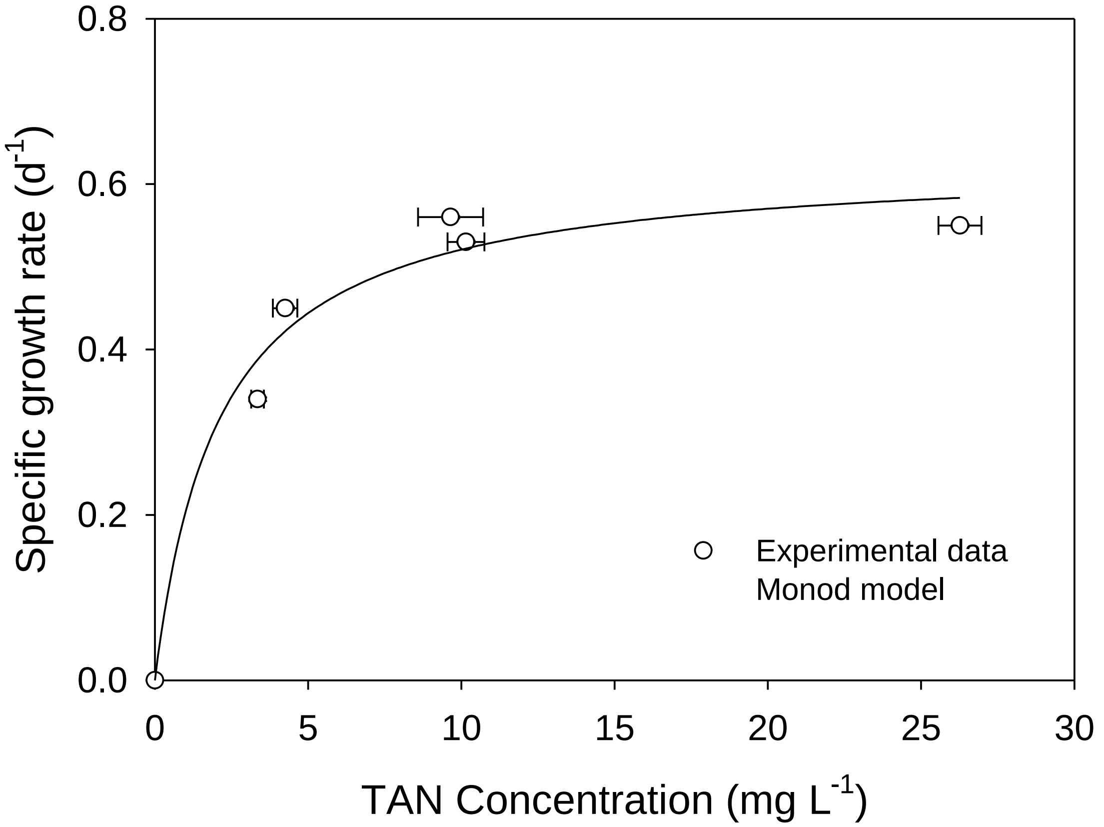

Figure 3

Figure

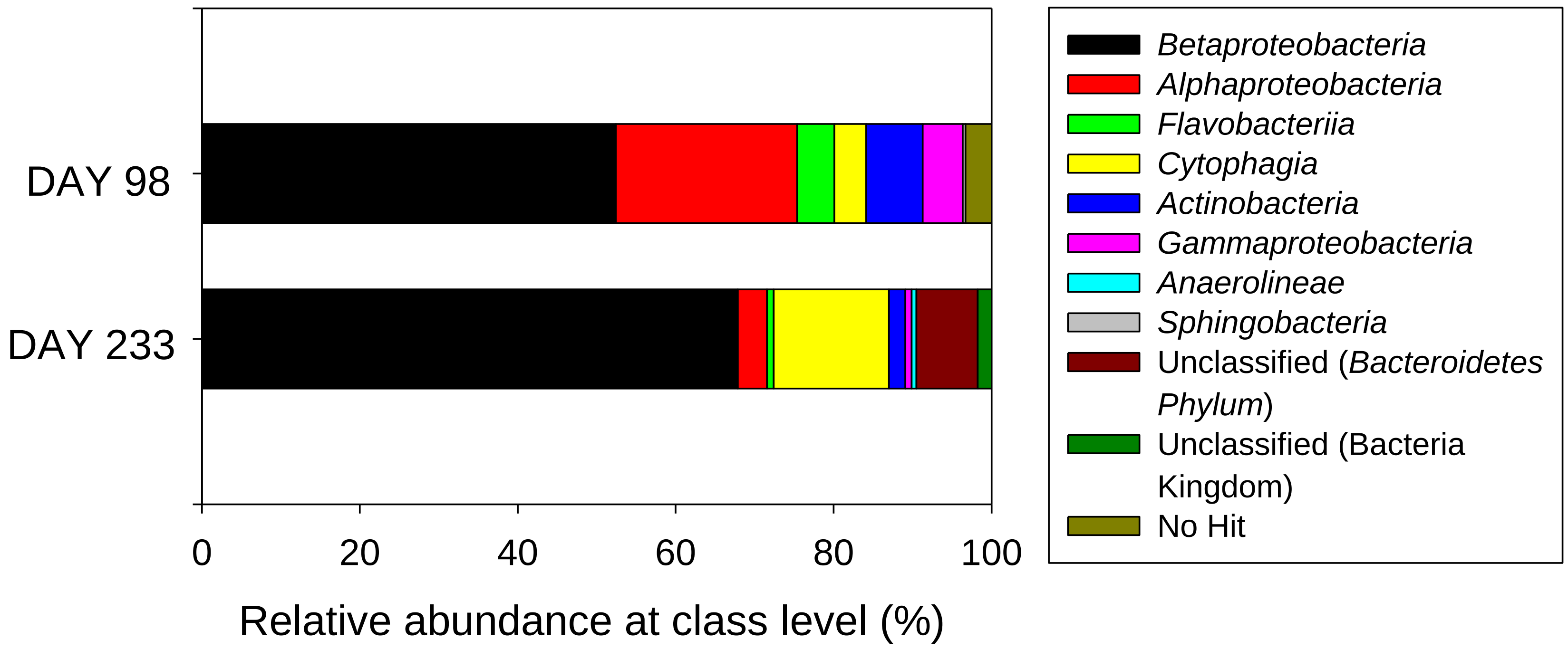

Figure 4

Figure

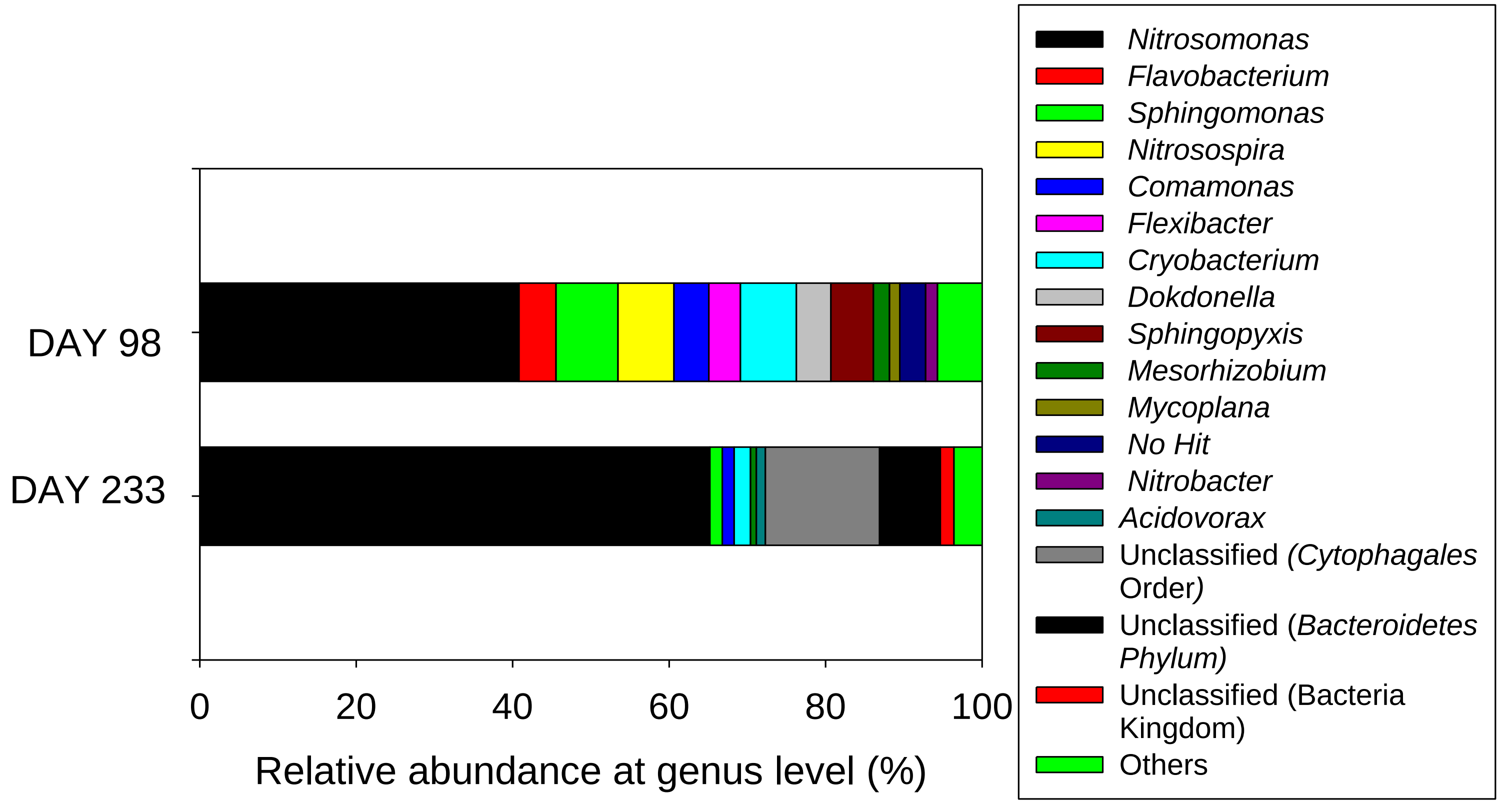

Figure 5

Figure

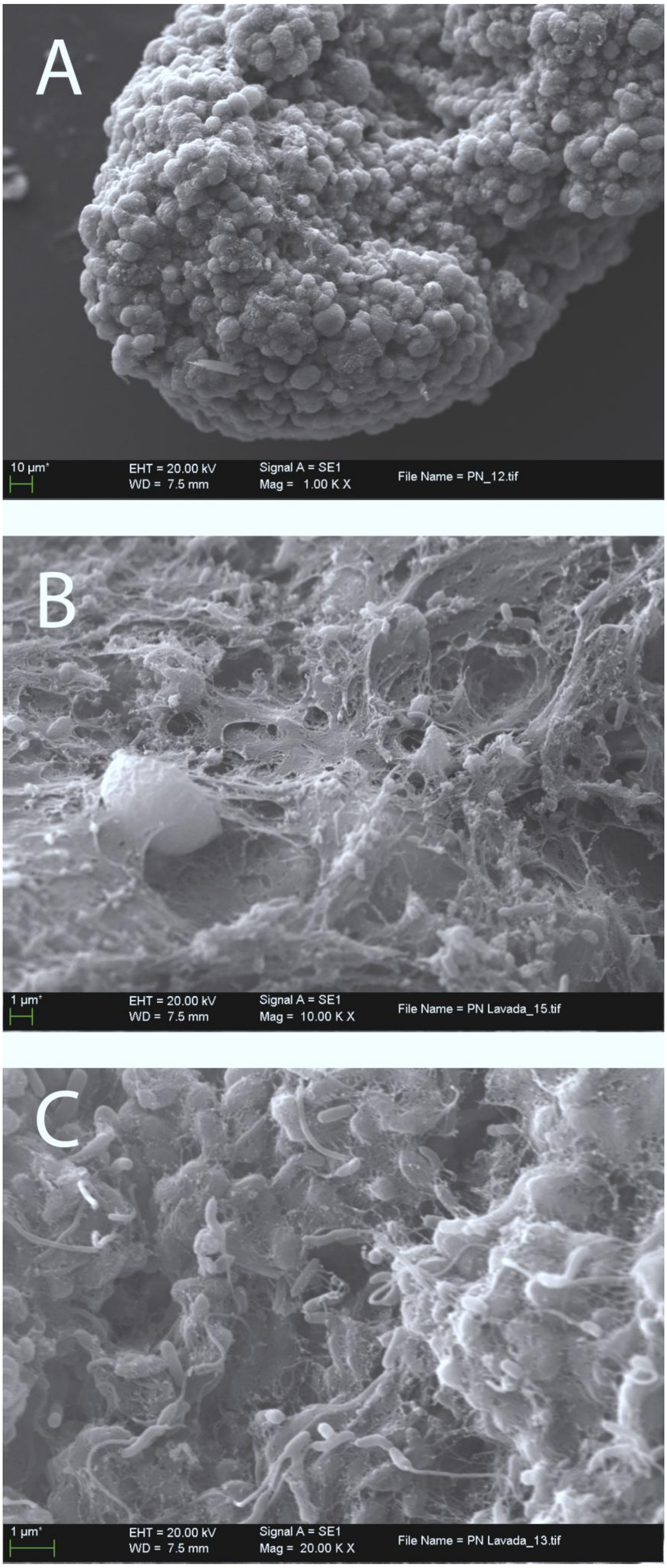

Figure 6