Simultaneous nitritation and *p*-nitrophenol removal using aerobic granular biomass in a continuous airlift reactor

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Abstract

The chemical and petrochemical industries produce wastewaters containing ammonium and phenolic compounds. Biological treatment of these wastewaters could be problematic due to the possible inhibitory effects exerted by phenolic compounds. The feasibility of performing simultaneous nitritation and *p*-nitrophenol (PNP) biodegradation using a continuous aerobic granular reactor was evaluated. A nitrifying granular sludge was bioaugmented with a PNP-degrading floccular sludge, while PNP was progressively added to the feed containing a high ammonium concentration. Nitritation was sustained throughout the operational period with ca. 85 % of ammonium oxidation and less than 0.3 % of nitrate in the effluent. PNP biodegradation was unstable and the oxygen limiting condition was found to be the main explanation for this unsteadiness. An increase in dissolved oxygen concentration from 2.0 to 4.5 mg O_2 L^{-1} significantly enhanced PNP removal, achieving total elimination. *Acinetobacter*

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genus and ammonia-oxidizing bacteria were the predominant bacteria species in the granular biomass.

Keywords: Nitritation, oxygen limitation, industrial wastewater, phenolic compounds, aerobic granules.

1. Introduction

Currently, biological nitrogen removal via nitrite route is considered as the technology with the cheapest costs and the lowest environmental foot-print for treating ammonium-rich wastewaters (Van Hulle et al, 2010). However, several industries such as the chemical, petrochemical, coke plant and refineries produce complex wastewaters containing both, ammonium and phenolic compounds (Morita et al., 2007; Milia et al., 2012). Very often, expensive physico-chemical treatments are advisable for the treatment of these industrial wastewaters, due to the potential inhibitory effect of phenolic compounds over microorganisms in the biological treatment (Oller et al., 2011), specifically the inhibition of the nitrification process by phenolic compounds (Hu et al., 2005; Liu et al., 2005; Morita et al., 2007).

A possible option for achieving this challenge with only biological treatments could be the technology of aerobic granules. The systems based on aerobic granular biomass are known to perform better in front of inhibitory or toxic compounds compared to activated sludge systems because granule architecture causes diffusion gradients contributing to protect sensitive bacteria (Adav et al., 2009; Maszenan et al., 2011). In this sense, some studies have shown the possibility of aerobic granules to perform simultaneous removal of ammonium and phenolic compounds (Liu et al., 2011; Suja et al., 2012).

Besides, the development and maturation of aerobic granules is performed, in general, in sequencing batch reactors (SBR) where high hydrodynamic stress is applied to form and to maintain the granules (Gao et al., 2011). Nevertheless, conventional batch operation is not advisable for treatment of phenolic compounds that usually exhibit inhibition by substrate (Martín-Hernández et al., 2009). To solve this problem, continuous operation could be a suitable option, since the concentration of the recalcitrant compounds in the reactor is expected to be low due to the high removal efficiency, reducing their toxic effect in the reactor. This high removal efficiency could be attained from the beginning of the operation by performing a controlled enrichment of the specific degrading biomass, i.e. by feeding this kind of compounds progressively during the start-up (Martín-Hernández et al., 2009).

In this sense, the development of a biological treatment dealing simultaneously with phenols and ammonium-rich wastewaters is of paramount importance.

In this study, *p*-nitrophenol (PNP) was selected as model compound of the nitrophenols family that are widely used, as reflected by their inclusion in the list of High Volume Production Chemicals (OECD, 2008). So that, the simultaneous nitritation and PNP removal using a continuous aerobic-granular airlift-reactor is investigated and evaluated for the treatment of an industrial wastewater containing PNP and a high concentration of ammonium . Granular biomass was characterized in terms of size, density, sludge volumetric index, settling velocity and contents of exopolymeric substances (EPS). Fluorescence in-situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM) was also performed for identification and quantification of the predominant bacteria species and to determine their spatial arrangement in the granules.

2. Materials and Methods

2.1. Experimental set-up and reactor conditions

A glass airlift reactor with a working volume of 2.6 L was used. The internal diameter of the down-comer was 62.5 mm. The riser had a height of 750 mm and an internal diameter of 42.5 mm, and it was at 8 mm from the bottom of the down-comer. Figure S1 in supporting information depicts a schematic diagram of the experimental set-up. Compressed air was supplied through an air diffuser placed at the bottom of the reactor. The reactor was equipped with dissolved oxygen (DO) (Crison DO 6050) and pH probes (Crison pH 5333) that were connected to a data monitoring system (Crison Multimeter 44). The temperature in the reactor was maintained using a temperature controller coupled with a belt-type heating device (Horst, Germany). pH of the reactor was maintained by a regular addition of $NaHCO_3$ into the reactor. Feeding to the reactor was made with a membrane pump (ProMinent Gamma/L). Air flow-rate in the reactor was regulated by rotameter (Aalborg, USA) with a range of 125-500 mL min^{-1}. Samples were regularly withdrawn from 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® (PVDF) prior to analysis. The operational conditions in the reactor during the experimental period were: Temperature of 30 ± 1 ºC and pH of 8.1 ± 0.4.

2.2. Wastewater composition

The airlift reactor was fed with synthetic wastewater containing 3.6 g L^{-1} NH_4Cl (950 ± 25 mg $N\text{-}NH_4^+$ L^{-1}) and the following compounds and micronutrients

(concentrations are expressed in mg L^{-1}): CH_3COONa, 48.0; glucose, 12.5; sucrose, 11.9; $CaCl_2 \cdot 2H_2O$, 88.0; KH_2PO_4, 41.0; NaCl, 176.0; $MgCl_2 \cdot 7H_2O$, 198.0; $FeSO_4 \cdot 7H_2O$, 4.0; $MnSO_4 \cdot H_2O$, 3.0; $ZnSO_4 \cdot 7H_2O$, 4.0; $CuSO_4 \cdot 5H_2O$, 2.0; and H_3BO_3, 0.02; $CO(NH_2)_2$, 12.0 and yeast extract, 2.0. In addition, an increasing amount of PNP was added to the influent, for details see Section 2.4 and Figure S2 in supporting information.

2.3. Inoculum and reactor operation before bioaugmentation

The airlift reactor was inoculated with 1 L of granular biomass from a granular sequencing batch reactor (GSBR) at pilot scale treating low-strength wastewater for simultaneous carbon, nitrogen, phosphorus removal (Isanta et al., 2012). Then, the reactor was operated in continuous using a synthetic high-strength ammonium wastewater (950 ± 25 mg $N\text{-}NH_4^+$ L^{-1}) to obtain nitritation maintaining the DO / ammonium concentration ratio below 0.02 mg O_2 mg^{-1} N (Bartrolí et al. 2010). Prior to bioaugmentation, the reactor was performing partial nitritation, oxidising ca. 48% of ammonium to nitrite at 0.3 ± 0.1 g N L^{-1} d^{-1} of volumetric nitrogen loading rate (NLR_V). The average effluent concentrations were: ammonium, 505 ± 40 mg N L^{-1}; nitrite, 462 ± 40 mg N L^{-1} and nitrate, 2 ± 1 mg N L^{-1}. Just before bioaugmentation, the granular biomass characteristics were as follow: mean size (mm) 1.1 ± 0.7, settling velocity (m h^{-1}) 66 ± 27, sludge volumetric index (SVI_5) (mL g^{-1} TSS) 8 ± 2, ratio SVI_5/SVI_{30} 1.0 and biomass density (g VSS $L_{particle}^{-1}$) 370 ± 140.

2.4. Bioaugmentation and operational strategy

At day 0, the airlift reactor was bioaugmented with 500 mL (2 g VSS L^{-1}) of a floccular sludge from a SBR performing stable PNP degradation (Martín-Hernández et

al., 2009). The bioaugmented biomass was accounted to be 15 % of the total biomass inside the airlift reactor. Martín-Hernández et al. (2012) suggested using ca. 5 % w/w for a successful bioaugmentation and retention of specialised biomass in a SBR. Considering that the airlift reactor was operating in continuous and biomass wash out would be inevitable, 15% of bioaugmented biomass was added instead of the 5% used by Martín-Hernández et al. (2012). The bioaugmentation procedure was repeated twice, on days 7 and 14.

The microbial composition of the PNP-degrading floccular sludge was characterized through fluorescence in situ hybridization (FISH) coupled to confocal laser scanning microscope (CLSM) following the protocol developed by Suárez-Ojeda et al. (2011). The FISH-CLSM results allowed identification and quantification of *Arthrobacter* sp. (26±2 %) and genus *Acinetobacter* (31±10 %) as the PNP-degraders in the floccular sludge, whereas no hybridization was found for *Burkholderia* sp. and *Pseudomonas* spp.

The PNP concentration was progressively increased during the experimental period to minimise its potential toxic or inhibitory effect over the granular biomass. During the first 14 days, PNP concentration in the influent was of 5 mg PNP L^{-1}, then, it was increased to 10 mg PNP L^{-1} during the next 115 days, and finally it reached 15 mg PNP L^{-1} for the last 111 days of the experiment (the reader is kindly referred to the Figure S2 in supporting information for a graphical overview of the feeding strategy).

This feeding strategy was selected by two reasons: (i) the progressive increase of PNP should allow to ease biodegradation and to promote the growth of specific PNP-degrading bacteria into the granules, and (ii) the AOB inhibition/toxicity by PNP should be minimized.

2.5. Analytical methods

PNP was determined by High Performance Liquid Chromatography (HPLC) as described by Martín-Hernández et al., (2009). The ammonium as total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$), the nitrite as total nitrite nitrogen (TNN = $N\text{-}NO_2^-$ + $N\text{-}HNO_2$) and nitrate concentrations were measured as described by Bartrolí et al., (2010). Volatile suspended solids (VSS), total suspended solids (TSS) and sludge volumetric index (SVI) were determined using the procedure described in Standard Methods (APHA, 1998). The granular biomass was characterized in terms of size, granule density, settling velocity and EPS content. The size distribution of the granules was measured regularly by using image analysis with an optical microscope Zeiss Axioskop equipped with a video camera (iAi Protec). The digital image captured was further processed using Image-Pro Plus version 6.0 (Media Cybernetics, Inc.,). The procedure followed was (i) to convert the original image to black and white for image processing, (ii) to define the threshold in order to delimit the area of interest in the image (i.e. the granules) and (iii) to export the selected data with the software to a worksheet. For each mean size determination, at least 50 granules were used. The density of the granular biomass was determined using the Dextran Blue method described by Beun et al. (2002). The settling velocity was determined by placing individual granule in a column containing the described wastewater and measuring the time spent to drop a height of 40 cm (Bartrolí et al., 2010). The EPS were extracted from the granules using formaldehyde + NaOH according to Adav and Lee, (2011).

p-Nitrophenol (granular solid form, purity 99%) and ammonium chloride (purity 99.5%) were employed and supplied by Panreac (Spain) and Carl Roth (Germany),

respectively. All the chemicals and other reagents were purchased from Sigma-Aldrich (Spain) and the highest purities available were employed.

2.6. Determination of the oxygen half-saturation coefficient for PNP-degraders ($K_{O_{PNP}}$) and the ammonia-oxidising bacteria (AOB) inhibition coefficient for PNP ($K_{I_{PNP,AOB}}$)

$K_{O_{PNP}}$ represents the DO concentration at which the PNP biodegradation rate is half of its maximum value:

$$r = r_{max} \frac{[DO]}{[DO]+K_{O_{PNP}}} \quad \text{Eq. (1)}$$

where r is the PNP degradation rate (g PNP L^{-1} d^{-1}), r_{max} is the maximum PNP degradation rate (g PNP L^{-1} d^{-1}), [DO] is the DO concentration (mg O_2 L^{-1}) and $K_{O_{PNP}}$ is the oxygen half-saturation coefficient for PNP-degraders (mg O_2 L^{-1}).

In order to determine the $K_{O_{PNP}}$, a respirometric experiment was carried out following the procedure proposed by Guisasola et al. (2005). In brief, the procedure for $K_{O_{PNP}}$ determination is based on monitoring the drop in DO concentration in a respirometer when external aeration is stopped, and the biomass is consuming substrate (PNP in this case) without limitation. At this moment, the DO concentration in the liquid phase sharply decreased because of the oxygen consumption being linked to the substrate consumption. This oxygen consumption rate corresponded to the maximum oxygen uptake rate (OUR_{max}) assuming that no substrate limitations existed. It is essential to avoid any substrate limitation (except for oxygen) for a reliable $K_{O_{PNP}}$ estimation. The oxygen mass balance that describes this system is as follows:

$$\frac{d[\mathrm{DO}]}{dt} = k_L a^{sup} \cdot ([\mathrm{DO}^*] - [\mathrm{DO}]) - (\mathrm{OUR_{end}} + \mathrm{OUR_{max}}) \cdot \frac{[\mathrm{DO}]}{[\mathrm{DO}]+K_{O_{PNP}}} \quad \text{Eq. (2)}$$

where $k_L a^{sup}$ is the global oxygen mass transfer constant through the liquid-gas surface (d^{-1}), [DO^*] is the DO concentration at saturation (mg O_2 L^{-1}), [DO] is the DO concentration (mg O_2 L^{-1}), OUR_{end} is the endogenous OUR value (mg O_2 L^{-1} d^{-1}), OUR_{max} is the maximum OUR value (mg O_2 L^{-1} d^{-1}) and $K_{O_{PNP}}$ is the oxygen half-saturation coefficient for PNP-degraders (mg O_2 L^{-1}).

An open and aerated Liquid-Flow-Static (LFS) respirometer was employed. The volume of the respiration vessel was 1 L and it was magnetically stirred. pH, DO concentration and temperature were measured in the liquid phase through a DO probe (WTW-Cellox 325) and a pH probe (WTW-Sentix 81), which were connected via RS-232 with the PC to store and monitor the data. The respiration vessel was submerged in a thermostatic bath, and the temperature was maintained at 30.0 ± 0.5 °C. The pH was maintained at 8.0 ± 0.1 with controlled addition of HCl (1M) and NaOH (1M). The PNP-degrading biomass was a floccular sludge obtained from a SBR performing stable PNP biodegradation (Martín-Hernández et al., 2009).

The $K_{I_{PNP,AOB}}$ determination was carried out following the procedure described by Suárez-Ojeda et al. (2010). In brief, the percentage of inhibition was determined by comparing the OUR_{max} measured when a control pulse is added inside a respirometer, before (1^{st} pulse) and after (2^{nd} pulse) an inhibitor pulse according to:

$$\%\ \mathrm{Inhibition} = \left[\frac{\mathrm{OUR}_{\mathrm{max}}^{1^{\mathrm{st}}\ \mathrm{pulse}} - \mathrm{OUR}_{\mathrm{max}}^{2^{\mathrm{nd}}\ \mathrm{pulse}}}{\mathrm{OUR}_{\mathrm{max}}^{1^{\mathrm{st}}\ \mathrm{pulse}}}\right] \cdot 100 \quad \text{Eq. (3)}$$

Respirometric tests were performed to measure the AOB inhibition by PNP. Ammonium was used as a control substrate, and different concentrations of PNP 0.5, 2, 15, 25 and 30 mg L^{-1} were used as the inhibitory substrate. Each test consisted in three sequential pulses: a first pulse of 10 mg $N\text{-}NH_4^+$ L^{-1}, a pulse of PNP and finally a second pulse of 10 mg $N\text{-}NH_4^+$ L^{-1}added after the DO concentration return to its initial DO concentration value (the DO before the first control substrate was added). An example of one of these tests can be seen in Figure S3 (supporting information). The experiment set-up was similar to the previously described for the $K_{O_{PNP}}$ determination. The temperature was maintained at 30.0 ± 0.5 °C while the pH was maintained at 8.0 ± 0.1 with controlled addition of HCl (1M) and NaOH (1M). The enriched AOB culture was a floccular sludge obtained from a pilot plant performing stable full nitritation (Torà et al., 2012).

The AOB inhibition by PNP was modelled as a non-competitive inhibition as follows:

$$\%\ \text{Inhibition} = \left(1 - \frac{K_{I_{PNP,AOB}}}{K_{I_{PNP,AOB}} + [\text{PNP}]}\right) \cdot 100 \qquad \text{Eq. (4)}$$

where [PNP] is the PNP concentration (mg L^{-1}) and $K_{I_{PNP,AOB}}$ is the AOB inhibition coefficient for PNP (mg L^{-1}).

All parameters were estimated using MATLAB 7.5 (The Mathworks, Natick, MA, USA). The differential equation was solved using an explicit Runge-Kutta formula. Parameter estimation was carried out by using the Nelder-Mead Simplex search method.

2.7. FISH analysis

FISH coupled with CLSM was used to determine the fractions of PNP-degraders, of betaproteobacterial ammonia-oxidising bacteria (βAOB) and of nitrite-oxidising bacteria (NOB) in crushed granules. Sliced granules were used for determining the distribution of bacteria species along the granule geometry. Both analyses were performed at the end of the experimental period. Hybridization was carried out using probes targeting specific microorganisms for PNP-degraders (ACA652 and KO 02), βAOB (Nso190) and NOB (NIT3). The general probe consisting of equal parts of UNIV1390 and EUBmix was used for detection of all bacteria. The probes used for PNP-degraders were selected taking into account the characterisation of the PNP-degraders culture used for bioaugmentation following the methodology stated by Suárez-Ojeda et al. 2011 (see section 2.4. Bioaugmentation and operational strategy).

For biomass fractions quantification, the granular biomass was crushed using a mortar and a pestle and then, FISH procedure was followed. The quantification of the microbial populations was performed following a modification of the procedure described in Jubany et al., (2009), where 40-50 microscopic fields were analyzed, and a single z-position was selected based on the highest intensity for each sample.

For sliced granules, entire granules were embedded in paraffin wax before their sectioning with a microtome. Slices with a thickness of 3 μm were cut, and each single section was placed on the surface of poly-L-lysine coated microscopic slides prior to follow the FISH protocol. In order to obtain a better sliced granule staining, the amount of probe used in the hybridisation step was increased 4 to 5 times, depending on the total area of the sliced granule.

A Leica TCS-SP5 AOBS confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was used with a HC PL APO CS 63x1.25 oil objective and equipped with two He-Ne lasers and a hybrid detector.

3. Results and discussion

3.1. Performance of the granular airlift reactor for simultaneous nitritation and PNP removal

Before performing bioaugmentation, a batch test was carried out to assess the capacity of nitrifying granules to biodegrade PNP (data not shown). The test consisted on the addition of a pulse of ammonium and PNP in an aerated batch reactor. The results of this test showed that the nitrifying granular biomass was not able to biodegrade PNP. Moreover, PNP-degrading species in the nitrifying granules (section 2.7) was assessed through FISH-CLSM with negative results for genus *Acinetobacter* and for *Arthrobacter* sp. Consequently, the nitrifying granular airlift reactor was bioaugmented with floccular PNP-degrading biomass as explained in section 2.4.

The performance of nitritation and PNP biodegradation in the bioaugmented aerobic granular airlift reactor is shown in Figure 1. As can be seen in Figure 1.A, two periods can be distinguished regarding nitritation performance. In the first period from day-0 to day-185, the applied NLR_V was 0.4 ± 0.1 g N $L^{-1}d^{-1}$, while during the second period from day-185 onwards, an average NLR_V of 1.0 ± 0.1 g N $L^{-1}d^{-1}$ was applied.

In the first period, the reactor was performing stable nitritation with an average of 62 % of ammonium oxidation and less than 0.2 % of nitrate in the effluent with a DO concentration in the bulk liquid of 1± 0.5 mg O_2 L^{-1} (Figure 1.B). However, PNP biodegradation behaviour (Figure 1.C) was rather unstable.

On Figure 1.C, it can be seen that PNP was accumulated during the first 7 days up to 5 mg L^{-1}. On believe that PNP-degraders bioaugmented on day 0 were washed-out from the system, a new bioaugmentation event on the day 7 was performed resulting in total PNP biodegradation. However, on day 14 PNP was again accumulated to 4 mg L^{-1} (Figure 1.C), and another bioaugmentation event was carried out. On same day 14, PNP in the influent was increased to 10 mg L^{-1}. Once more, PNP was accumulated to 10 mg L^{-1} in the following 7 days, but then it decreased progressively to less than 1 mg L^{-1} on day 45, without any other bioaugmentation event. From day 45 to 60, PNP biodegradation was maintained between 60 and 95 %. However, nitritation decreased steadily from the initial 62 to 27 % (Figure 1.B).

As no operational changes were applied to the reactor, two possible reasons were figured out for explaining the behaviour of nitritation and PNP biodegradation processes in this period: i) AOB activity was inhibited by PNP and ii) AOB and PNP-degraders activities were limited by DO. The latter reason was assessed by increasing the DO concentration in the bulk liquid from 1.0 to 2.0 ± 0.5 mg O_2 L^{-1} on day 60. From this moment, nitritation was improved but remained unstable for the following 120 days. The effluent concentrations in this period were TAN: 50-400 mg N L^{-1}, TNN: 560-950 mg N L^{-1} and nitrate below 1 mg N L^{-1}, with a NLR_V at 0.42 g N L^{-1} d^{-1} (Figures 1.A and 1.B). The same can be described for PNP degradation, from day 60 to 180, PNP was cyclically accumulated. Consequently, the reactor performance until day 180 did not allow to distinguish which of these two hypotheses was correct.

To deepen the understanding of the process and to be able to discern between both hypotheses, two different respirometric experiments were carried out: (i) to determine

the AOB inhibition coefficient for PNP ($K_{I_{PNP,AOB}}$) and (ii) to determine the oxygen half-saturation coefficient for PNP-degraders ($K_{O_{PNP}}$).

To find out whether AOB activity was inhibited by PNP, $K_{I_{PNP,AOB}}$ was determined through respirometric experiments using an enriched AOB floccular sludge performing full nitritation. The results obtained from the respirometric experiment are presented in Figure 2.A. The estimated $K_{I_{PNP,AOB}}$ value was 7 ± 2 mg PNP L^{-1}. This value indicates the PNP concentration resulting in 50% inhibition of AOB activity. The results observed in Figure 1 show that TAN build up in the effluent occurred just after high PNP accumulation in the reactor (e.g. days 75-90, days 125-140 or days 150-170). As an example, on day 75, PNP accumulated to 10 mg L^{-1}, and TAN concentration in the effluent increased from 190 on day 75 to 380 mg N L^{-1} on day 95, this means a 20 % reduction in nitritation conversion. It might be expected that PNP concentrations higher than 7 mg PNP L^{-1} would cause AOB activity inhibition by more than 50%, but none of the TAN accumulation events was so significant. The reason that could explain this phenomenon is that granular biomass is better able to cushion the effect of an inhibitory compound than floccular sludge (Maszenan et al., 2011). Thus, the apparent $K_{I_{PNP,AOB}}$ value for AOB embedded in granules should be higher than the determined in this study for an AOB floccular sludge. Moreover, after 185 days of operation one might expect that the AOB were acclimated to the presence of PNP, this would also contribute to increase the apparent $K_{I_{PNP,AOB}}$ value of this AOB population compared to an AOB floccular sludge that has never been exposed to PNP.

To assess the second hypothesis, i.e. to establish whether AOB was outcompeting PNP degraders at the DO concentration imposed in the reactor, $K_{O_{PNP}}$ was determined

through a specific respirometric test (Figure 2.B). The estimated $K_{O_{PNP}}$ was 1.65 ± 0.01 mg O_2 L^{-1}. This value is close to that reported by Contreras et al. (2008) for phenol ($K_{O_{Phenol}}$= 1.84 mg O_2 L^{-1}), higher than that reported for AOB (0.74 mg O_2 L^{-1}) by Guisasola et al.(2005) and much higher than that reported for heterotrophic bacteria of 0.20 mg O_2 L^{-1} (Henze et al., 2000). Therefore, the supplied DO concentration at 2.0 mg O_2 L^{-1} throughout the first 185 days of the operational period was insufficient for maintaining a stable full PNP degradation because the aerobic PNP biodegradation was limited by DO concentration. This finding confirmed the hypothesis about oxygen limiting condition to explain the instability of PNP removal in the reactor.

In view of these results, from day 185 onward the second period of the airlift operation was started with a DO concentration in the bulk liquid of 4.5 ± 0.5 mg O_2 L^{-1} aim was to obtain full PNP biodegradation that will further eliminate AOB inhibition. Complete and stable PNP degradation was achieved on day 185 onward, allowing to increase both, PNP-loading rate and NLR_V to 16 PNP L^{-1} d^{-1} and 1.0 g N L^{-1} d^{-1}, respectively. It is interesting to highlight that despite the simultaneous PNP biodegradation, this NLR_v is comparable to the reported in the literature for the treatment of high-strength ammonium wastewater at 30ºC but, without containing any phenolic compound: Yamamoto et al. (2011) 0.7-2.6 g N L^{-1} d^{-1}, Okabe et al. (2011) 1.0-1.8 g N L^{-1} $d^{-1}$and Bartrolí et al. (2010) 0.75-6.1 g N L^{-1} d^{-1}.

Moreover, nitrite oxidation was always prevented due to the strong oxygen limiting conditions imposed in the reactor for NOB. Although since day 60 onwards, DO concentration in the reactor was between 2.0 and 4.5 mg O_2 L^{-1}, the TAN concentration was always kept in great excess. Therefore, the DO/TAN concentration ratio in the reactor was very low (between 0.04 and 0.16 mg O_2 mg^{-1} N), outcompeting

the NOB in the granules, as already demonstrated in previous studies (Bartrolí et al., 2010; Jemaat et al., 2013). This fact is later confirmed with the FISH analyses, where occurrence of *Nitrobacter* sp. was very low (see section 3.2 for further details).

The biomass concentration in the reactor was maintained between 2.4 to 3.2 g VSS L^{-1} (Figure 3.A). On the first 30 days of the experimental period, an increase of effluent biomass concentration was observed up to a maximum of 250 mg VSS L^{-1} (Figure 3.A). This would be related to the washout of a fraction of the bioaugmented PNP-degrading floccular sludge that could not be retained in the reactor. From day 50 to the end of the experimental period, the biomass concentration in the effluent was always maintained at a low value 39 ± 20 mg VSS L^{-1}. The good settling ability of the granular biomass is demonstrated by the low SVI_5 values obtained, ranging from 9 to 13 ± 2 mL g^{-1} TSS (Figure 3.B). Only SVI_5 was depicted in Figure 3.B since SVI_{30} values were identical to SVI_5 values throughout the whole operational period, and consequently the SVI_{30}/SVI_5 ratio was always one.

3.2. Characteristics of the granular biomass and identification of dominant species in the granules

Granule size, biomass density and settling velocity were not significantly affected throughout the operational period (Figures 3.B and 3.C). The biomass density decreased during the first 100 days of operation from 390 g VSS $L_{particle}^{-1}$ until a stable value of 210 g VSS $L_{particle}^{-1}$. This could be explained by the development and attachment of heterotrophic biomass able to degrade PNP in the form of filamentous structures that inevitably will decrease the compactness of granular biomass (see section 3.2). All the

parameters were in the range expected for granular sludge, according the review performed by Gao et al. (2011).

EPS content in granules was unchanged during the first 100 days of operation (Table 1). However, a slight increase was observed at the end of the reactor operation. An increase of EPS concentration in this period could be linked to: i) the increase in DO concentration through an increase in the airflow-rate applied to the reactor and consequently, higher shear stress conditions were applied to the granules and ii) the increase in the applied loading rate. Both actions could stimulate the microbial activity and thus, increase production of EPS (Tay et al., 2001a). In general, higher concentrations of PS than PN were observed during the operational period (Table 1). The high PS content in EPS was also reported in several studies with nitrifying granules (Tay et al., 2001b; Liu and Tay, 2004). High PS content was noted to facilitate cell to cell adhesion and strengthen the microbial structure through a polymeric matrix (Adav et al., 2008).

Besides the determination of DO concentration as an important parameter enhancing the overall performance of this biofilm reactor, another key feature for maintaining simultaneous nitritation and PNP biodegradation at long-term is the development, retention and attachment of the PNP-degraders over the nitrifying granules. To observe the morphological changes in the nitrifying granules throughout the experimental period, a magnifying glass was used to obtain pictures of the granules on days 0, 112 and 220 (Figure S4, in supporting information). On the beginning, the nitrifying granules possessed a smooth surface and regular granular shape (Figure S4.A). After 112 days of operation and also on day 220, the outer surface of nitrifying granules were covered with filamentous structures (Figures S4.B and S4.C), probably

linked to the growth of bacteria related to PNP biodegradation. FISH analysis was performed at the end of the operational period to aiming to confirm this supposition.

The results from FISH -CLSM identified that only *Acinetobacter* genus and βAOB were the predominant populations at the end of the experimental period, whereas *Nitrobacter* sp. was detected at very low occurrence and *Arthrobacter* sp. was never detected in the samples. It was quantified that *Acinetobacter* genus accounted for about 50±14%, βAOB for 49±20% and *Nitrobacter* sp for 1±1%. *Acinetobacter* genus detected in the granules was one of the dominant bacteria populations in the bioaugmented PNP-degraders (section 2.4), responsible for PNP biodegradation. These results seem to indicate that only *Acinetobacter* genus and not *Arthrobacter* sp. was capable of being retained in the granular airlift reactor. The low occurrence of *Nitrobacter* sp confirms that NOB were outcompeted by AOB in the granules due to the imposed DO/TAN concentration ratio that guarantees strong oxygen limiting conditions for NOB, as already demonstrated in previous studies (Bartrolí et al., 2010).

Figure 4 shows the spatial distribution of the different bacterial populations in a sliced granule. *Acinetobacter* genus seems to proliferate in the outer layer (Figure 4.A), whereas βAOB tends to locate in the inner layers of the granule (Figure 4.B) and *Nitrobacter* sp. is almost undetectable (Figure 4.C). As in Figure S4, some filaments are also visible in Figure 4. These results confirm not only the development of the specific heterotrophic bacteria for PNP biodegradation, but a microbial stratification in the aerobic granules following different metabolic activities. This stratification is probably the key for understanding the high capacity of the AOB population in the granules to bear significant PNP concentrations in the bulk liquid.

3.3 Practical implications of this study

The results show that a continuous biological treatment dealing simultaneously with p-nitrophenol removal and nitritation of ammonium-rich wastewaters is feasible by using aerobic granules. However, for achieving complete nitrogen removal a following step must be coupled to this reactor, which could be done either through heterotrophic denitrification or using anammox bacteria.

There is no doubt that the cheapest process, from both the economical and the environmental point of view, will be partial nitritation plus anammox. However, the complexity of real industrial wastewaters is a tremendous challenge and sometimes an obstacle for the anammox process due to the inhibition of the anammox bacteria by several pollutants (Jin et al., 2013).

In this sense, a more robust process towards the presence of phenols in the effluent of the nitrification reactor could be a heterotrophic denitrification as pointed out by De la Torre-Velasco et al. (2013). The key points of this integrated process will be: i) The use of aerobic granular biomass for the simultaneous treatment of phenols and ammonium-rich wastewater for producing a suitable effluent for the heterotrophic denitrification, ii) the use of a suitable [DO]/[TAN] ratio to achieve and to maintain stable nitritation, avoiding oxygen limiting conditions for getting a stable removal of phenols.

4. Conclusions

Simultaneous nitritation and PNP biodegradation was successfully demonstrated in a single reactor using aerobic granular sludge. Nitritation, with less than 0.3% of N-NO_3^-, was steadily maintained for more than 185 days despite unstable PNP biodegradation

Bioaugmentation enhanced the formation of a diversified microbial consortium (AOB and PNP-degraders) over the granules. The oxygen limiting conditions were found to be the key factor affecting the stability of PNP biodegradation, as complete PNP degradation was achieved and stably maintained when DO concentration was increased to a non-limiting value.

The granular characteristics were not significantly affected during the simultaneous nitritation and PNP biodegradation.

5. Acknowledgements

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Table 1

Table 1. Content of extracellular polymeric substances in aerobic granular sludge during the simultaneous nitritation and PNP biodegradation.

Time (day)	Polysaccharides (PS) (mg g^{-1} VSS)	Protein (PN) (mg g^{-1} VSS)	EPS (PS +PN) (mg g^{-1} VSS)	Ratio PS/PN
0	22 ± 2	22 ± 1	44 ± 3	1.00
100	27 ± 3	14 ± 1	41 ± 4	1.92
224	36 ± 6	24 ± 1	60 ± 7	1.50

Figure captions

Figure 1. Performance of a granular nitrifying airlift reactor treating a high-strength ammonium wastewater containing *p*-nitrophenol. (A) Volumetric nitrogen loading rate (NLR_V) and DO profile, (B) Nitritation performance, (C) *p*-nitrophenol biodegradation during the experimental period. Arrows indicate bioaugmentation events took place on days 0, 7 and 14, respectively.

Figure 2. (A) Experimental and simulated AOB inhibition values by PNP; (B) Dissolved oxygen profile obtained in determining the oxygen half saturation coefficient, $K_{O_{PNP}}$ for PNP-degraders. Experimental conditions in both experiments were temperature, 25 °C; pH, 8.0 ± 0.1.

Figure 3. (A) Volatile suspended solid (VSS) concentrations and SVI values; (B) Granule size, biomass density and settling velocity in the granular airlift reactor during the operational period.

Figure 4. FISH image of a sliced granule collected at the end of the experimental period (Bar = 100 µm). A) Yellow: *Acinetobacter* genus (ACA652); B) Red: βAOB (Nso190); C) Green: *Nitrobacter* sp. (NIT3); D) Blue: All bacteria (EUBmix+UNIV1390); E) Merge image. Centre of the granules is on the upper left corner.

Figure 1

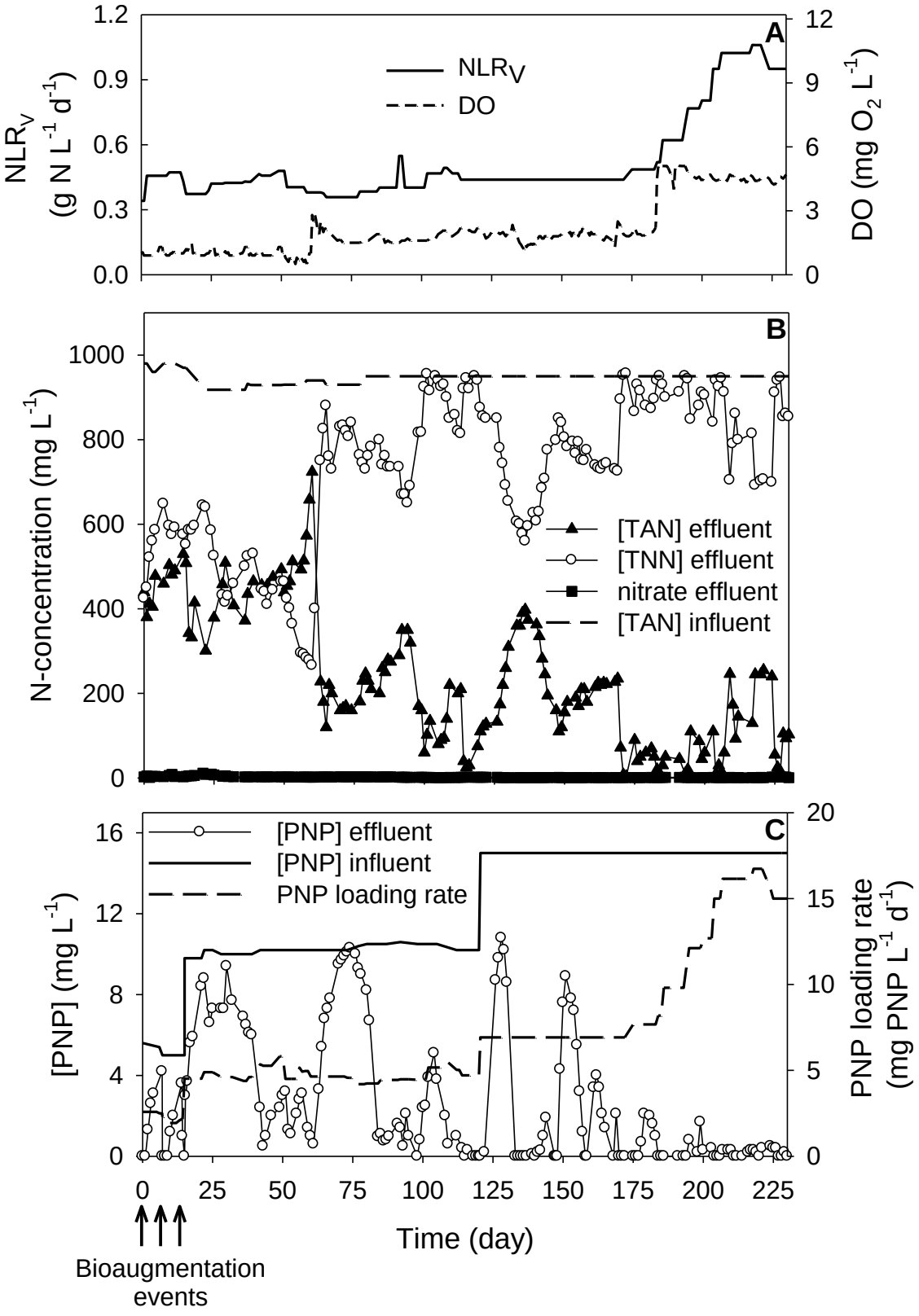

Figure 1.

Figure 2

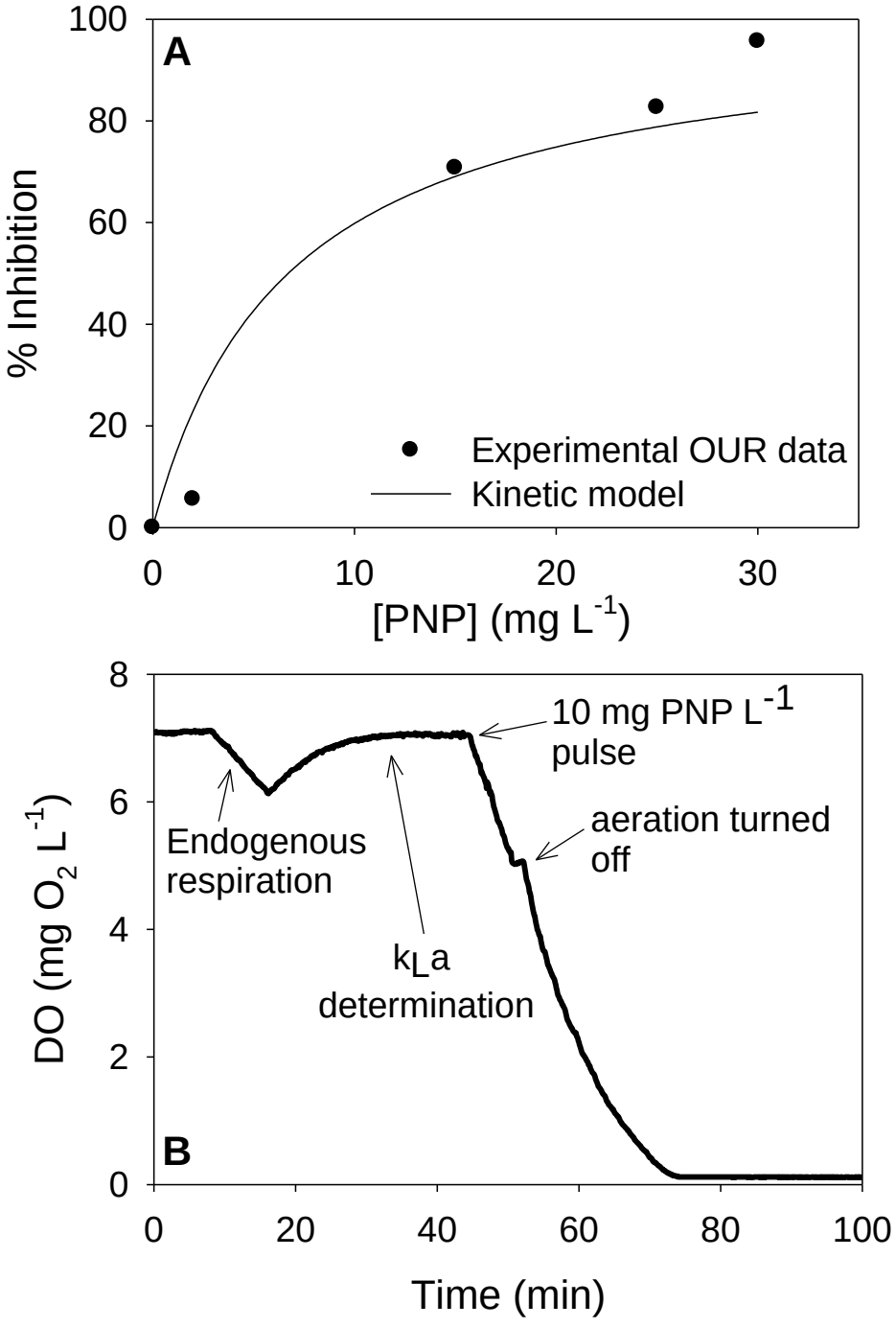

Figure 2.

Figure 3

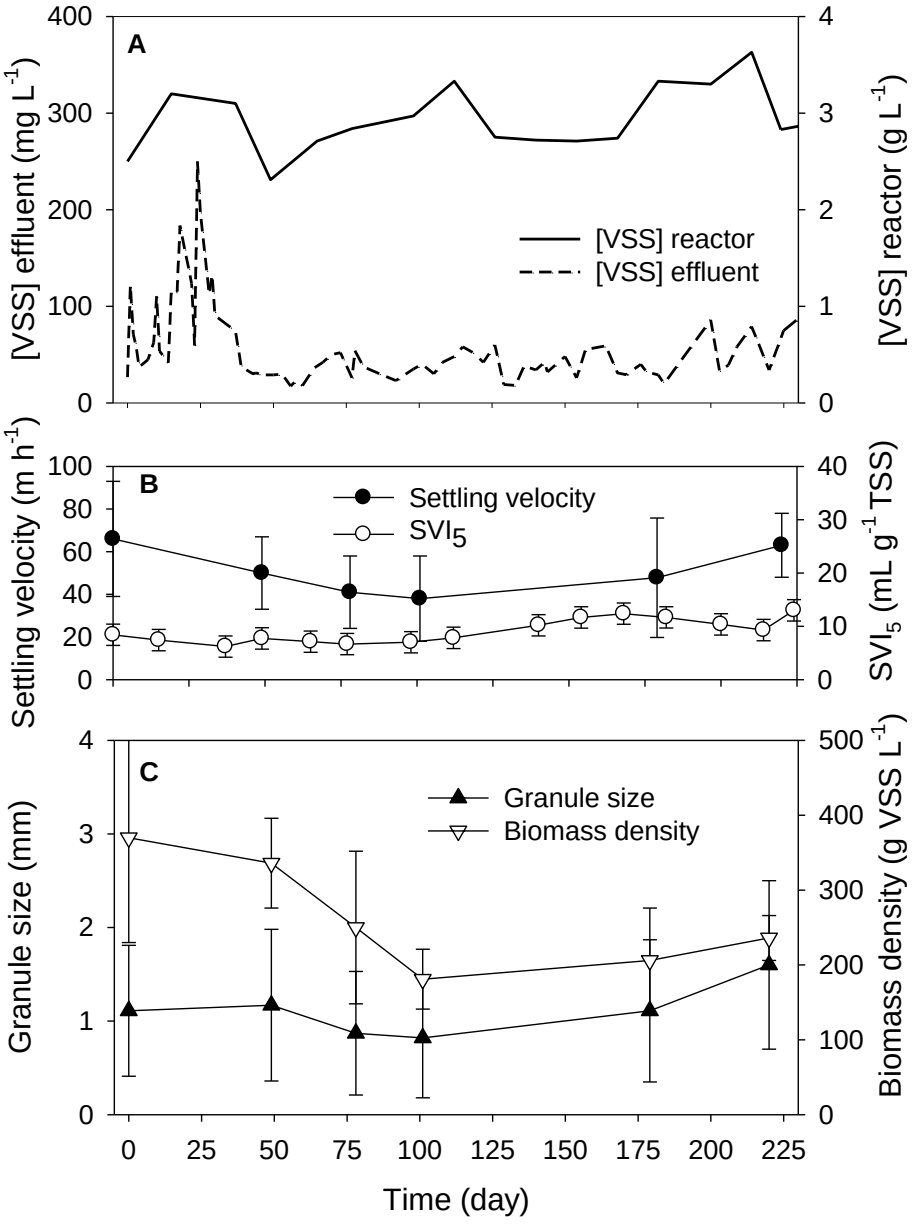

Figure 3.

Figure 4

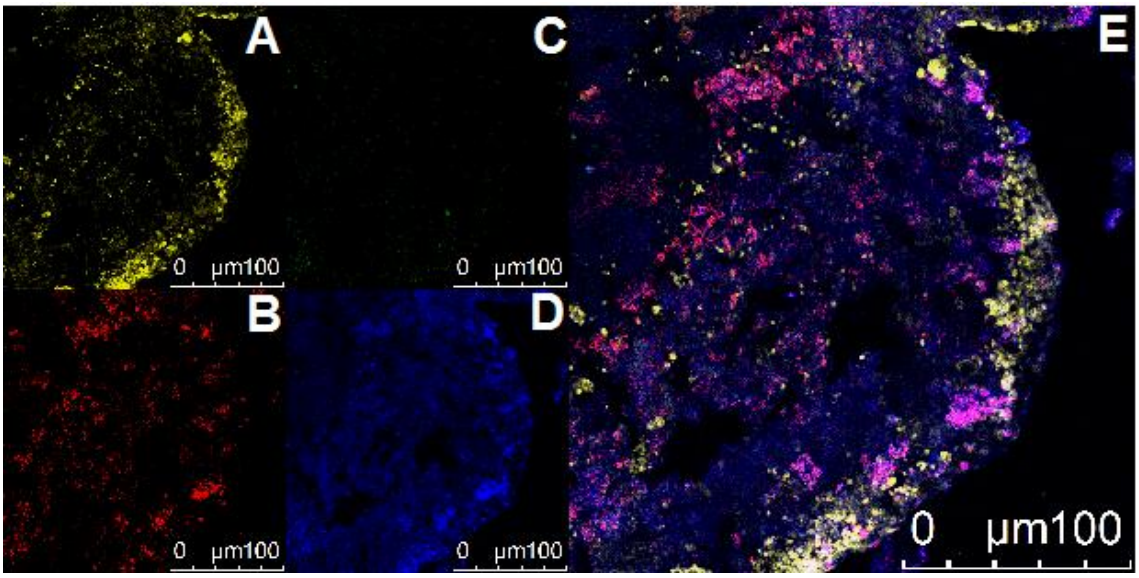

Figure 4.