Long-term impact of salinity on the performance and microbial population of an aerobic granular reactor treating a high-strength aromatic wastewater

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Abstract

The effect of salinity over granular biomass treating a mixture of aromatic compounds (phenol, *o*-cresol and *p*-nitrophenol) was evaluated in a continuous airlift reactor. To mimic an industrial wastewater, increasing concentrations (from 2.0 to 29.0 g salts L^{-1}) of a mixture of salts ($MgSO_4$, NaCl, KCl, $CaCl_2$ and $NaHCO_3$) were introduced in the influent. The gradual salinity increase led to a good acclimation of the biomass obtaining complete biodegradation of the aromatic compounds and no accumulation of metabolic intermediates. However, a deterioration of the morphology of aerobic granules with a complete loss of granulation after 125 days was produced at 29.0 g salts L^{-1}. At that moment, anaerobic granules were added to promote granulation and after 50 days new aerobic granules were formed. These new aerobic granules remained stable for more than 100 days at the highest salinity condition with 100% removal of the mixture of aromatic compounds.

Keywords: aerobic granulation; *Sphingobium* genus; *Devosia* genus; phenolic compounds; salts

1. Introduction

Industrial wastewaters are composed by numerous pollutants constituting, therefore, a very complex matrix difficult to be treated. The effluents produced by agro-industries, paper-making, petrochemicals, pharmaceuticals, landfill leachates, chemical manufacturing, pesticides and herbicides industries can contain significant amounts of inorganic dissolved salts (Lefebvre and Moletta, 2006). For instance, carbonates (1-16 g L^{-1}) (Olmos et al., 2004), chlorides (1-40 g L^{-1}) (Manekar et al., 2013; Olmos et al., 2004) and sulphates (10-25 g L^{-1}) (Manekar et al., 2013). Besides, most of these wastewaters also contain organic compounds, particularly toxic and recalcitrant pollutants, such as aromatic compounds: phenol (0.3-31 g L^{-1}) (Bai et al., 2010; Kim and Kim, 2003; Olmos et al., 2004) and *o*-cresol (0.03-6 g L^{-1}) (Kim and Kim, 2003; Olmos et al., 2004).

Industrial wastewaters are often treated by physico-chemical processes. However, these technologies have serious drawbacks (Al-Khalid and El-Naas, 2011; Kim and Ihm, 2011): (i) high costs due to the required conditions of temperature and pressure and the use of some chemicals, (ii) incomplete degradation of the recalcitrant/toxic organic compounds and (iii) generation other hazardous by-products (secondary pollutants). 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 widely known to be inhibited by aromatic compounds (Kim and Ihm, 2011) and also to be affected by high salinity. Inorganic salts can influence negatively over the structure and settling properties of microbial flocs (Lefebvre and Moletta, 2006). This fact is related to the density of salty water, which is higher than that of freshwater, thus creating greater resistance to decantation through higher buoyant forces (Lefebvre and Moletta, 2006).

To overcome the inhibition caused by organic compounds and the detrimental effect of salts, a promising alternative to activated sludge systems is the application of reactors with aerobic granular biomass (Gao et al., 2011). The application of aerobic granules allows retaining slow growing microorganisms and protects them from high concentrations of pollutants due to the diffusion gradient produced through the granule (Gao et al., 2011), favouring gradual adaptation to stressing conditions.

To the best of the authors knowledge, aerobic granules have been usually used for treating a single toxic/recalcitrant compound and a single salt (usually NaCl) (Li and Wang, 2008; Pronk et al., 2013; Taheri et al., 2012; Wan et al., 2014). Moreover, these studies have been carried out in sequencing batch reactors (SBRs). However, conventional batch operation is not the best option for the treatment of toxic/recalcitrant compounds, since the occurrence of high concentrations of these compounds at the beginning of a cycle can generate inhibitory conditions for the microorganisms. In this sense, continuous reactors would be a better option compared to SBRs in order to prevent these inhibitory effects since the bulk liquid concentration of the

toxic/recalcitrant compound in a continuous reactor is expected to be low if the removal efficiency is high.

Therefore, taking into account this lack of practical information about the performance of aerobic granular reactors treating complex wastewaters containing mixtures of aromatic compounds and salts in continuous reactors, this study aims to evaluate the long-term effect of salinity on the biodegradation of a mixture of aromatic compounds by aerobic granules in a continuous airlift reactor.

2. Materials and methods

2.1. Reactor

A glass airlift reactor with a working volume of 2.6 L was utilized in this study. 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. Compressed air was supplied through an air diffuser placed at the bottom of the reactor at an upflow velocity of 0.2-0.3 cm s^{-1}. Airflow rate in the reactor was regulated manually between 150 to 250 mL min^{-1} by a rotameter (Aalborg, USA) and it was enough to ensure an appropriate flow in the airlift reactor. The reactor was equipped with dissolved oxygen (DO) (Crison DO 6050), temperature (Crison Pt1000) and pH probes (Crison pH 5333) that were connected to a data monitoring system (Crison Multimeter 44). DO was manually maintained between 4.0 and 6.0 mg O_2 L^{-1} along the reactor performance. The DO concentrations were increased stepwise: 4.0, 4.5, 5.0 and 6.0 mg O_2 L^{-1}, when the salinity increased: 2.0, 6.5, 13.0 and 29.0 g salts L^{-1},

respectively. These concentrations were selected (i) to maintain a concentration over 4 mg O_2 L^{-1} to achieve successful aerobic biodegradation of *p*-nitrophenol (Jemaat et al., 2013) and (ii) to avoid a decreased oxygen transfer to the liquid phase due to the decrease in the oxygen maximum solubility at high salinity conditions, which can lead to DO limitations. A Programmable Logic Controller (PLC) coupled to a Supervisory Control And Data Acquisition (SCADA) system regulated temperature, pH and feeding. pH was maintained at 8.0 ± 0.2 by a regular addition of $NaHCO_3$ whereas temperature in the reactor was maintained at 30 ± 0.5 ºC using a temperature controller coupled with a belt-type heating device (Horst, Germany). Feeding to the reactor was made with a membrane pump (ProMinent Gamma/L).

2.2. Granular biomasses

Aerobic granular sludge from a continuous airlift reactor performing simultaneous biodegradation of p-nitrophenol, phenol and o-cresol was used as inoculum. This reactor exhibited complete biodegradation of the aromatic compounds and their metabolic intermediates at an organic loading rate of 0.61 g COD L^{-1} d^{-1} in presence of a concentration of salts of 2.0 g L^{-1}. Stable granules were obtained throughout the long-term operation (more than 250 days). Some of the granular biomass characteristics were as follows: average granule size of 220 ± 20 µm, sludge volumetric index (SVI) at 30 min of 26 ± 1 mL g^{-1} of Total Suspended Solids (TSS) and SVI_{30}/SVI_5 ratio of 1.0. More information can be found on Ramos et al. (submitted).

On day 125 of the reactor operation, 260 mL (10 % of the working volume of the reactor) of anaerobic granular biomass was added to the reactor to promote granulation.

The anaerobic granular biomass was obtained from a full-scale internal circulation (IC) reactor treating an industrial wastewater and their characteristics were SVI_{30} of 8.0 ± 0.5 mL g^{-1} TSS, SVI_{30}/SVI_5 of 1.0, average granule size of 1100 ± 10 µm and 87 % of biomass with a diameter higher than 0.2 mm. The wastewater treated by these anaerobic granules had a salinity of 8.0 g salts L^{-1}.

2.3. Wastewater composition and operational conditions

The airlift reactor was continuously fed with synthetic wastewater. The organic carbon source was maintained constant along the experiment and it was composed by several aromatic compounds: phenol (1340 ± 50 mg COD L^{-1} equivalent to 563 ± 21 mg L^{-1}), *p*-nitrophenol (565 ± 30 mg COD L^{-1} equivalent to 350 ± 17 mg L^{-1}) and *o*-cresol (240 ± 10 mg COD L^{-1} equivalent to 95 ± 6 mg L^{-1}). Sucrose and glucose were added along the whole operational time as co-substrate in a *p*-nitrophenol:(glucose+sucrose) ratio of 0.4 (as COD). Therefore, the total concentration of organic matter in the influent was 2890 ± 240 mg COD L^{-1}, where the aromatic compounds represented around 75 % of the COD. The composition of the micronutrients in the synthetic wastewater was maintained constant along the whole experimental period and it was composed as follows (expressed as mg L^{-1}): 88 of $CaCl_2x2H_2O$; 106 of NH_4Cl; 41.0 of KH_2PO_4; 176.0 of NaCl; 198.0 of $MgCl_2x7H_2O$; 4.0 of $FeSO_4x7H_2O$; 3.0 of $MnSO_4xH_2O$; 4.0 of $ZnSO_4x7H_2O$; 2.0 of $CuSO_4x5H_2O$; 0.02 of H_3BO_3; 12.0 of $CO(NH_2)_2$ and 1.0 of yeast extract.

Along the reactor operation, the biomass was exposed to increasing salts concentrations, maintaining constant the concentrations of the organic carbon sources. The salinity in

the wastewater was increased along the operational period by adding a mixture of salts: $MgSO_4$, NaCl, KCl, $CaCl_2$ and $NaHCO_3$, which were added in equal parts. In this sense, the synthetic wastewater mimicked a complex matrix as in real industrial wastewaters. The salinity was measured as conductivity, which gave the ionic strength of the wastewater.

The operational period was divided in two phases. During phase I (the first 125 days of operation), the salinity was increased stepwise: 2.0, 6.5, 13.0 and 29.0 g salts L^{-1}, which corresponded to conductivities of 1.4, 4.5, 9.0 and 20.0 mS cm^{-1}, respectively. During phase I, the biomass of the effluent was recycled back to the reactor. Therefore, the effluent of the reactor was collected each day and allowed to settle down during 30 min. Later the supernatant was removed and the concentrated biomass was returned to the reactor. Phase II started on day-125 after the addition of anaerobic granules and lasted other 125 days. During phase II, the recycle of biomass was stopped.

2.4. Samples and analytical methods

Samples were regularly withdrawn from the influent and 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. Phenol, *o*-cresol, *p*-nitrophenol and metabolic intermediates (quinoline, 1,2,4-benzenetriol, hydroquinone, catechol, resorcinol and *p*-nitrocatechol) 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 remained isocratic until 20 min. The injection volume was 20 µL and the maximum pressure in the column was approximately 290,000 hPa. Conductivity was measured with a CRISON conductivity device (micro CM 2100) with independent temperature probe and conductivity cell and the data was expressed at 25 °C to the method 2510 B (APHA, 1999). Total organic carbon (TOC) was measured with an OI Analytical TOC Analyser (Model 1020A) equipped with a non-dispersive infrared (NDIR) detector and with a furnace maintained at 680 °C. COD was calculated from the TOC experimental data using the theoretical relationship between COD and TOC in the combustion reaction of each organic compound.

Total and volatile suspended solids (TSS and VSS, respectively) were determined according to Standard Methods 2540 D and 2540 E (APHA, 1999).

The granular biomass was characterized in terms of sludge volumetric index (SVI), morphology, size and extrapolymeric substances (EPS). SVI_5 and SVI_{30} were determined using the 2710 D procedure described in Standard Methods (APHA, 1999). The size distribution and biomass size was measured by a laser particle size analysis system (Mastersizer 2000, Malvern instruments). Granules morphology was measured by using image analysis with an optical microscope (Zeiss Axioskop equipped with a video camera (iAi Protec)). EPS were extracted from the granules using formaldehyde and NaOH (Adav & Lee, 2011) and the polysaccharides and protein content in the EPS were measured using sucrose and bovine serum albumin as standards, respectively.

2.5. Microbial diversity analysis

Identification of the microbial population was performed using next-generation sequencing at day-25 (d-25) and day-250 (d-250) of the reactor operation. Also, the anaerobic granules used to promote granulation were analysed (RoA).

Total genomic DNA of the aerobic granular biomass was extracted and purified using a PowerBiofilm™ DNA Isolation Kit (MoBio Laboratories, USA); in accordance with the manufacturer's instructions. Paired-end sequencing of the extracted DNA was performed on Roche 454 GS-FLX+ 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 details can be found in supporting information. Biodiversity analysis and phylogenetic classification were performed with the methodology explained in detail in supporting information. Relative abundances of reads were determined by taxonomic level. Indices of biological diversity were calculated for d-25, d-250 and RoA libraries (Table S1 and Figures S1, S2 and S3 of the supporting information) indicating all libraries were comparable in terms of abundance percentages and that good coverage of diversity was reached.

3. Results and discussion

The operation of the airlift reactor was divided in two different phases, before and after the addition of anaerobic granules. In both periods, the reactor treated a wastewater composed by 563 ± 21 mg L^{-1} of phenol, 350 ± 17 mg L^{-1} of *p*-nitrophenol and 95 ± 6 mg L^{-1} of *o*-cresol, which were equivalent to 1340 ± 50, 565 ± 30 and 240 ± 10 mg

COD L^{-1}, respectively. During phase I (before the addition of anaerobic granules), the salinity was increased stepwise from 2.0 to 29.0 g salts L^{-1} (equivalent to 1.4-20.0 mS cm^{-1}, respectively). In this phase, the biomass contained in the effluent was recycled back to the airlift. At the beginning of phase II, the airlift was seeded with anaerobic granules to improve the granulation. During phase II, the salinity was maintained at 29.0 g salts L^{-1} (equivalent to 20.0 mS cm^{-1}) and the recycle of biomass from the effluent was stopped.

3.1. Impact of salinity on the morphological properties of the aromatics-degrading aerobic granules

At the beginning of this study (day-25, 2.0 g salts L^{-1}), bimodal peaks were observed in the particle size distribution analysis carried out through Mastersizer (Figure 1A), indicating that there were two types of bio-aggregates in the reactor. These two types of bio-aggregates can be also seen in Figure S4A (in supporting information). The average particle diameter was 220 ± 20 µm but 69 % per volume of particles were lower than 200 µm and the rest of particles were higher than 200 µm. SVI_{30} and SVI_{30}/SVI_5 ratio were 26 ± 2 mL g^{-1} and 1, respectively. These values indicate that biomass could be considered as aerobic granules although a significant part of the particles were below the typical size range of aerobic granules (de Kreuk et al., 2007). From day-25 onwards, salinity was increased stepwise and this increase caused a change in the morphological properties of the aerobic granules. On day-75, after 50 days at 6.5 g salts L^{-1}, the bimodal particle distribution disappeared and the average particle diameter decreased (160 ± 10 µm) (Figure 1B). In fact, only 23 % per volume of particles were higher than

200 µm. The loss of granules and the increase of floccular biomass can be seen comparing Figure S4A and S4B (in supporting information). Moreover, SVI_{30} and SVI_{30}/SVI_5 ratio also worsened (28 ± 1 mL g^{-1} and 0.67, respectively) but these values yet indicated a very good settleability of the biomass. This trend was confirmed at higher salinities. On day-100, after 25 days at 13.0 g salts L^{-1}, the bimodal particle distribution had completely disappeared (Figure 1C) and the average particle diameter decreased to 120 ± 10 µm. On day-125, after 25 days at 29.0 g salts L^{-1}, the particle distribution was completely unimodal (Figure 1D) with an average particle diameter of 110 ± 10 µm. These particle sizes distributions and the average particle diameter did not correspond to an aerobic granular biomass but to a floccular biomass. However, 16 % per volume of particles were still higher than 200 µm and SVI_{30} and SVI_{30}/SVI_5 ratio were 26 ± 3 mL g^{-1} and 0.56, respectively. Therefore, after 125 days at high salinity, the biomass had lost most of its granular morphology although it retained a very good settleability.

In spite of the loss of granules integrity, the preservation of good settleability of the bio-aggregates could be related to the high salinity, which produces the increase in water density, leading to the wash out (turbid effluent) of lighter flocs while bigger flocs remain in the reactor (Moussa et al., 2006). Throughout phase I, the biomass of the effluent was settled during 30 min, recovered and returned to the reactor. However, a small part of the biomass (the non-sedimentable part) was lost. Therefore, most of the biomass was recovered but the smallest and lightweight flocs were lost. Another explanation of the good settling capacity of the bio-aggregates could be the effect of salts acting as nuclei for bacterial aggregation. During phase I, the VSS/TSS ratio of the biomass in the reactor decreased from 62 to 23 % suggesting a possible effect of the

salts as nuclei for bacterial aggregation (Liu et al., 2015). However, this aggregation by the salts could not be enough to form mature granules at high salinities (higher than 2.0 g salts L^{-1}) and treating simultaneously aromatic compounds.

In spite of the good settleability, the aromatics-degrading biomass became floccular and its granular character was lost. This transformation seemed to be caused by the high salinity of the wastewater, but other studies treating readily biodegradable substrates found the opposite effect since a high salinity (33-50 g NaCl L^{-1}) produced a compaction of the aerobic granules (Li and Wang, 2008; Pronk et al., 2013). The difference between these studies and this one lies in the kind of organic matter treated. The formation of aerobic granules from floccular biomass or the maintenance of mature aerobic granules treating mixtures of aromatic compounds, containing specifically *p*-nitrophenol, it is not easy task. For instance, Suja et al. (2012) failed to reach a stable aerobic granular reactor at long-term treating aromatic compounds. They suggested that *p*-nitrophenol might inhibit many microbial species involved in granulation.

In order to promote granulation at high salinity conditions, the strategy of seeding the reactor with mature anaerobic granules was tested. This strategy, with different variations, was carried out in the past by other authors: (i) Linlin et al. (2005) studied the formation of aerobic granules consuming acetate by seeding their aerobic sequencing batch reactor (SBR) with anaerobic granules from an industrial upflow anaerobic sludge blanket reactor. These anaerobic granules were the sole inoculum of the aerobic SBR and they were basically disintegrated in the first days after inoculation and then, they played a role as nuclei for a subsequent granulation of the aerobic bacteria formed in the SBR. (ii) Muda et al. (2010) inoculated a SBR under alternating anaerobic/aerobic conditions with both, an aerobic floccular biomass able to degrade a

textile mill wastewater and an anaerobic granular biomass as nuclei for granulation. After 70 days of operation, they achieved mature granules with both, aerobic and anaerobic activities.

The strategy followed in this study can be considered a mix of the above exposed since mature anaerobic granules from an IC reactor were seeded on day-125 to the aerobic airlift reactor where there was an aerobic floccular biomass able to degrade aromatic compounds at high salinity conditions. The seeded anaerobic granules corresponded to a volume fraction of 10 % of the working volume of the airlift reactor. Just after the addition, on day-130, a bimodal particle size distribution was again obtained (Figure 2A). In this distribution, the average diameter was 280 μm but there were clearly two different types of bio-aggregates, 75 % in volume were the flocs present in the airlift reactor before the addition of the anaerobic granules (with an average diameter around 100 μm) and 25 % in volume corresponded to the anaerobic granules added (with an average diameter around 1000 μm). At this moment, there was still no improvement in the aerobic granulation. However, on day-175, after 50 days of the addition (Figure 2B), the average particle diameter increased up to 380 ± 100 μm, being 42 % per volume of particles higher than 200 μm. This result showed that the strategy of seeding anaerobic granules was valid to improve granulation of aerobic floccular biomass because the increase of both, the average diameter and the number of bio-aggregates higher than 200 μm was the result of the growth of new aerobic granules. On day-190, after 65 days of the addition (Figure 2C), the average diameter reached its maximum value (410 ± 40 μm) as well as the percentage of particles higher than 200 μm (49%). The reactor was operated during two more months to demonstrate the stability of the aerobic granulation achieved with this new strategy. In fact, on day-250, after 100 days of the addition of

anaerobic granules, the morphological characteristics of the aerobic granules remained stable (Figure 2D) contrary to phase I after long-term exposition at high salinity conditions. The bio-aggregates on day-250, after more than 100 days at high salinity conditions (29.0 g salts L^{-1}), can be considered aerobic granular biomass (see figure S4C in supporting information) with an average granule diameter of 370 ± 180 µm, a SVI_{30} of 4 ± 1 mL g^{-1} TSS and a VSS/TSS ratio of 0.14.

As stated by Verawaty et al. (2012), the most feasible mechanism to explain the success for aerobic granulation in phase II was the use of anaerobic granules added on day-125 which acted as nuclei for the attachment of the aerobic floccular biomass, developing a stable and mature aerobic granules able to degrade a mixture of aromatic compounds at high salinity conditions.

Moreover, EPS were determined in the aerobic granular biomass at low (27 ± 4 g g^{-1} VSS at day-25 and 2.0 g salts L^{-1}) and high salinity (61 ± 13 g g^{-1} VSS at day-250 and 29.0 g salts L^{-1}) conditions. The content of polysaccharides (PS) and protein (PN) was 2.3-fold higher at high salinity (PS: 37 ± 6 g g^{-1} VSS and PN: 25 ± 8 g g^{-1} VSS) than at low salinity conditions (PS: 17 ± 5 g g^{-1} VSS and PN: 10 ± 2 g g^{-1} VSS). This fact could indicate an overproduction of EPS as a response to high salinity. This observation has been reported in other studies; where the aerobic granulation at high salinity can be related to an increase in the EPS content (Taheri et al., 2012; Wan et al., 2014). EPS would have an important role in facilitating adherence of bacteria during formation of granules and/or be linked to the protection against the osmotic stress produced by the salts.

3.2. Impact of salinity on aerobic biodegradation of aromatic compounds

The removal capacity of the airlift granular reactor was basically unaffected by the increase of the salinity (Figure 3). During the first 125 days (Phase I), the organic loading rate (OLR) was maintained at 0.56 g COD L^{-1} d^{-1} and the percentage of COD removal was, in average, 96 ± 3% in spite of the progressively increase of salinity up to 29.0 g salts L^{-1}. There were only two periods with a lower removal capacity than the stated before during phase I, during the first 25 days and from day-50 to day-60. In both cases, there was a small accumulation of a metabolic intermediate (catechol) and a decrease of the percentage of COD removal. The first 25 days corresponded to the start-up period whereas the second period (day-50 to day-60) corresponded to an overpass of the maximum capacity of the reactor. No other metabolic intermediates (1,2,4-benzenetriol, hydroquinone, resorcinol and *p*-nitrocatechol) nor a primary aromatic compound (phenol, *o*-cresol and *p*-nitrophenol) were accumulated in both periods. In contrast, during phase I, the granulation worsened, as explained in the previous section, which means that salinity affected the cell-to-cell interactions needed for bio-granulation but not the capacity of the cells to remove the aromatic compounds. Probably, the removal capacity of the system was preserved because the biomass concentration was maintained high (4-5 g VSS L^{-1}) by recycling part of the biomass contained in the effluent.

After the addition of anaerobic granules, the biomass recycling was stopped and this caused the loss of a significant part of the bio-aggregates with the smallest size. Those smallest bio-aggregates were possibly composed by active aromatics-degrading bacteria and their lost caused a temporary accumulation of catechol from day-150 to day-175. Therefore, from day-150, OLR was stepwise decreased from 0.56 to 0.21 g COD L^{-1} d^{-1}

to remove the accumulated *p*-nitrophenol and catechol. After that, the removal of aromatic compounds remained totally stable with COD removal percentages close to 100%, no accumulation of intermediates and an applied OLR of 0.21 g COD L^{-1} d^{-1}.

3.3. Impact of salinity on microbial population

A pyrosequencing analysis was performed to determine the microbial population at class and genus level of the aerobic granules at low (Figure 4A) and high salinity (Figure 4B) conditions and also the microbial population of the anaerobic granules used to seed the reactor on day 125 (Figure S5 in supporting information). In the aerobic granules at low salinity (Figure 4A), Alphaproteobacteria, Cytophagia, "No Hit" and Betaproteobacteria were the main classes identified; representing 97 % of the reads. At genus level, *Sphingobium* (33 %), *Cytophaga* (21 %), "No Hit" (17 %) and *Comamonas* (14 %) were mainly detected. In the aerobic granules at high salinity (Figure 4B), the main classes were Alphaproteobacteria, "No Hit", Flavobacteria and Actinobacteria, representing 95 % of the reads. At genus level, *Devosia* (46 %), "Not Hit" (19 %), "Unknown" (19 %), *Sphingomonas* (6 %) and *Muricauda* (4 %) were detected.
From the comparison of these results, it can be concluded that most of the main classes and especially the genera changed from low to high salinity conditions. At class level, Cythophagia and Betaproteobacteria disappeared at high salinity while Alphaproteobacteria became the most abundant class at these conditions. This fact could be related to the diverse range of metabolic capacities Alphaproteobacteria has, such as: (i) partner-switching mechanism and signal transduction pathways controlling the the regulation of the general stress response (Francez-Charlot et al., 2015) and (ii) DNA

uptake (including transformation, transduction and conjugation, resulting in gaining additional genetic information) (Le et al., 2014) that allow this class to adapt and survive in stressing conditions, such as high salinity.

At genus level, the most abundant genera at low salinity (*Sphingobium*, *Cythophaga* and *Comamonas*) have been previously described as degraders of aromatic compounds in aerobic conditions (Fu et al., 2014; Jiang et al., 2004; Watanabe et al., 1998). This fact corroborates the high removal capacity of the reactor at low salinity (Figure 3). However, these genera disappeared at high salinity and *Sphingomonas, Muricauda* and especially *Devosia* became the most abundant genera. These three genera have been previously described as halotolerant bacteria (Hube et al., 2009; Lefebvre et al., 2006; Wan et al., 2014) and *Devosia* and *Sphingomonas* also as genera with aromatics–degrading capability (Fredrickson et al., 1999; Kiesel et al., 2007).

The microbial population of the anaerobic granules added on day-125 was also analysed to understand if it played a meaningful role in the changes of bacterial populations between low and high salinity conditions. At class level, “No hit”, Nitrospira, Deltaproteobacteria, Cytophagia, “Unknown” and Anaerolineae represented 81 % of the reads. At genus level, “No hit” (39 %), “Unknown (12)”, *Syntrophobacter* (10 %), *Magnetobacterium* (9 %) and *Cytophaga* (5 %) were the most abundant. In view of these results, it seems that the addition of the anaerobic granules was not the main reason to explain the changes produced in the microbial population in phase II. In any case, the main genera found in the aerobic granules at high salinity (*Devosia*, *Sphingomonas* and *Muricauda*) were not detected neither in the aerobic granules at low salinity nor in the anaerobic granules. Probably, they were present but at very low levels.

Another significant question is the role of the microbial populations in the aerobic granulation. It seems that EPS producing-bacteria play a significant role in the formation of aerobic granules (Zhu et al., 2015). In this sense, genera of EPS producing-bacteria were found in both aerobic granules at low salinity (*Sphingobium*; (Zhang et al., 2011)) and high salinity (*Sphingomonas*; (Zhang et al., 2015)). In the case of the aerobic granules at high salinity, the most abundant genus was *Devosia* (46 % of the reads). *Devosia* genus belonged to the order Rhizobiales, which includes a variety of bacteria able to produce EPS (Kaci et al., 2005). Therefore, *Devosia* genus could have a role in the aerobic granulation at high salinity conditions.

Therefore, the results of the pyrosequencing showed a change in the microbial populations of the aerobic granules treating a mixture of aromatic compounds at high salinity (29.0 g salts L^{-1}) compared to low salinity (2.0 g salts L^{-1}). In this sense, different microbial populations can be involved in the removal of aromatic compounds and aerobic granulation depending of the salinity degree.

4. Conclusions

The increase of salinity caused the loss of the aerobic granules integrity in an airlift reactor treating a mixture of aromatic compounds (phenol, *o*-cresol and *p*-nitrophenol). New mature aerobic granules were formed after the addition of anaerobic granules as nuclei for granulation. The biodegradation of the aromatic compounds was successful achieved in spite of the loss of the granulation. The microbial populations changed with the increase of the salinity conditions, being *Sphingobium*, *Cythophaga* and *Comamona*

the most abundant genera at low salinity and *Devosia*, *Sphingomonas* and *Muricauda* the most abundant genera at high salinity.

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FIGURE CAPTIONS

Figure 1. Bio–aggregates size distribution during phase I (before the addition of anaerobic granules). A: at day-25 (2.0 g salts L^{-1}), B: at day-75 (6.5 g salts L^{-1}), C: at day-100 (13.0 g salts L^{-1}) and D: at day-125 (29.0 g salts L^{-1}).

Figure 2. Bio–aggregates size distribution during phase II (after the addition of anaerobic granules) maintain constant the salinity at 29.0 g salts L^{-1}. A: at day-139, B: at day-175, C: at day-189 and D: at day-250.

Figure 3. Effect of the salinity over the biodegradation of aromatic compounds throughout the operation of the airlift reactor. A: organic loading rate (OLR) and salinity applied, B: COD concentration in the effluent and COD removal, C: COD concentrations in form of *p*-nitrophenol, *o*-cresol and phenol in the effluent and D: COD concentrations in form of metabolic intermediates in the effluent.

Figure 4. Microbial diversity at class and genus level of the biomass in the airlift reactor at: (A) low salinity level (day-25, 2.0 g salts L^{-1}) and (B) high salinity level (day-250, 29.0 g salts L^{-1}). 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 per sample.

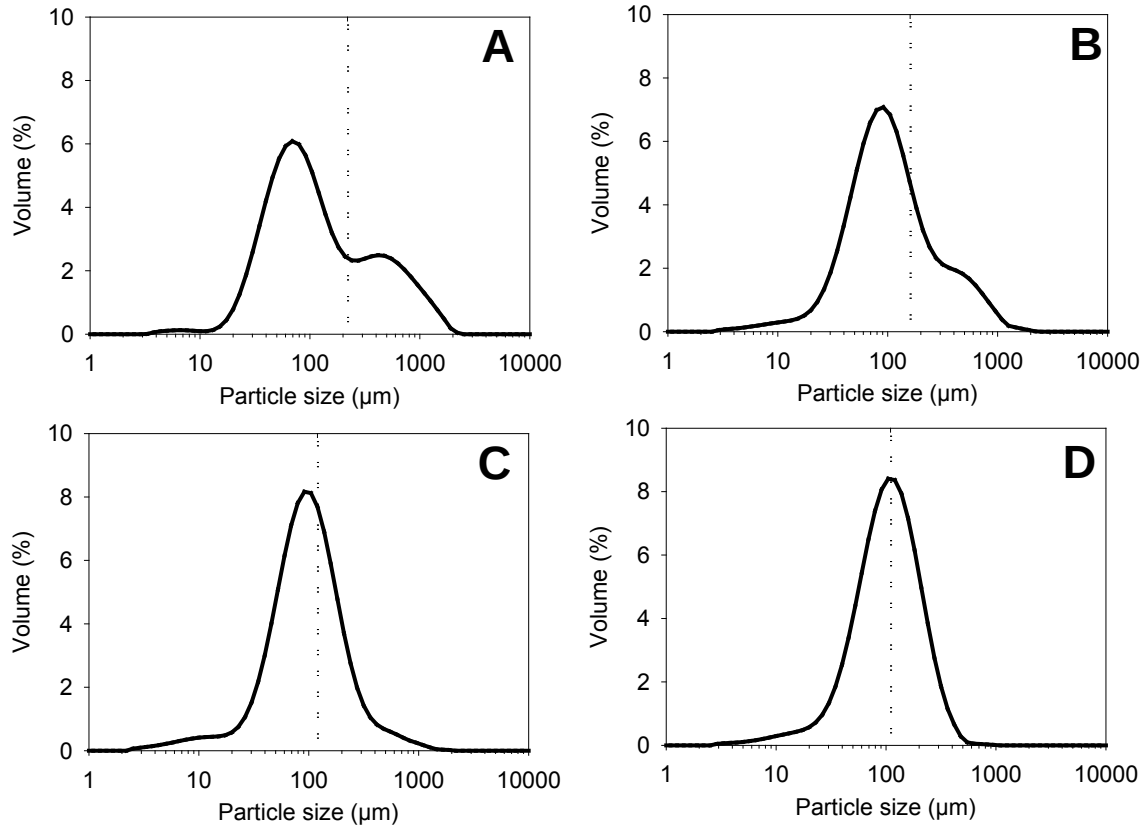

Figure 1

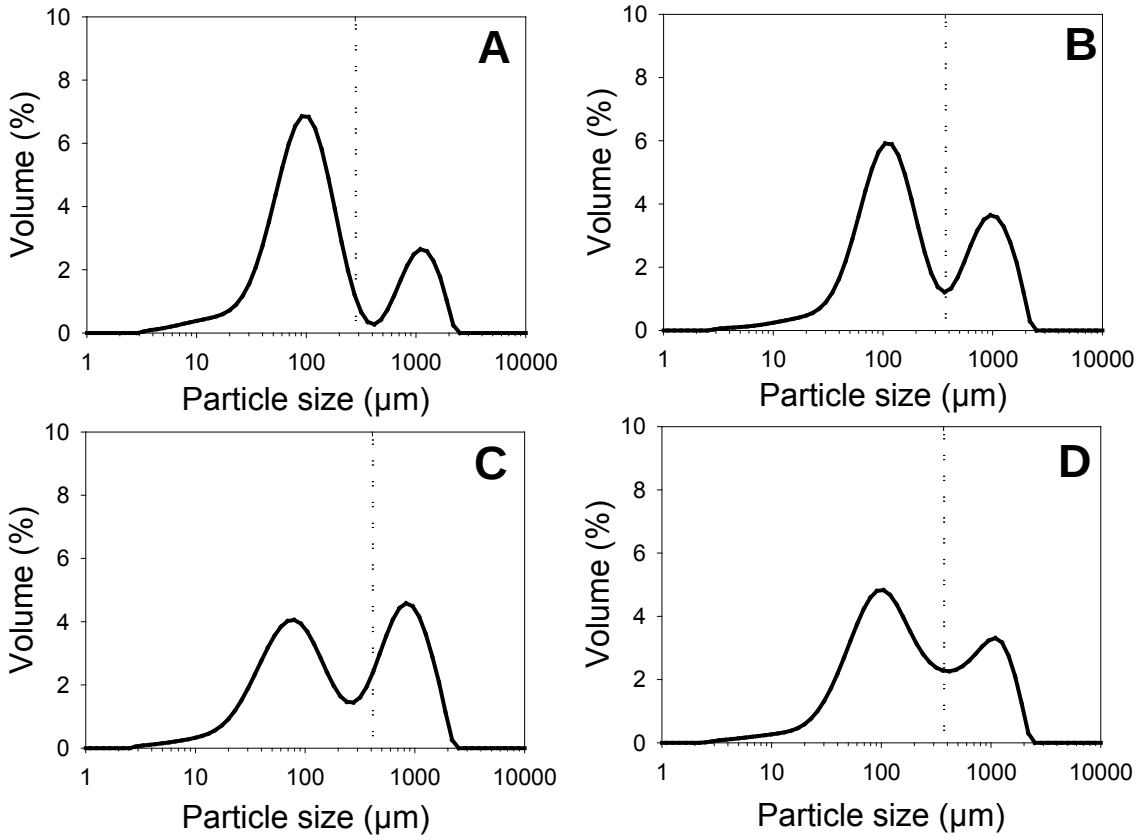

Figure 2

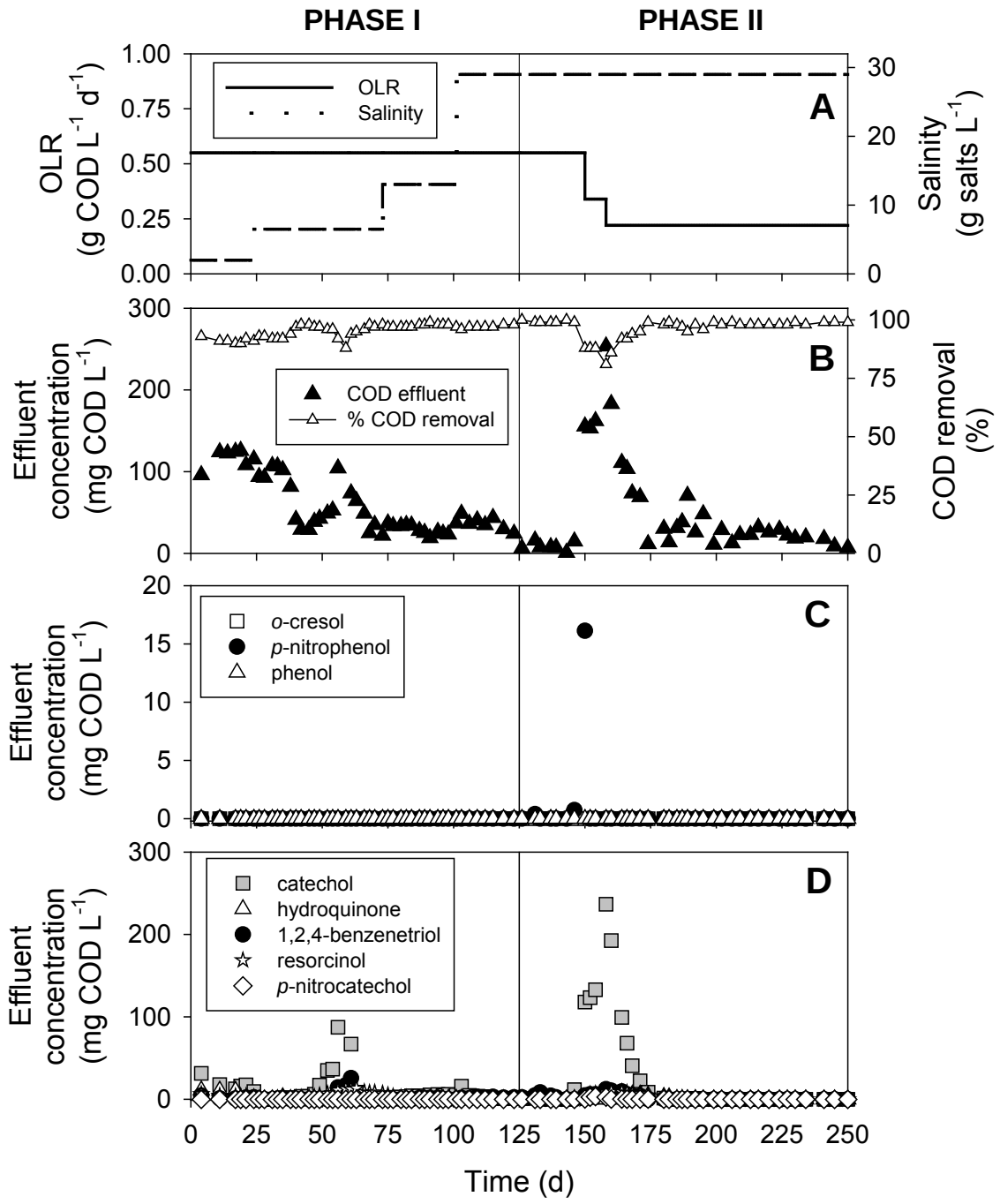
PHASE I
PHASE II
OLR
Salinity
A
OLR (g COD L-1 d-1)
Salinity (g salts L-1)
COD effluent
% COD removal
B
Effluent concentration (mg COD L-1)
COD removal (%)
o-cresol
p-nitrophenol
phenol
C
catechol
hydroquinone
1,2,4-benzenetriol
resorcinol
p-nitrocatechol
D
Time (d)

Figure 3

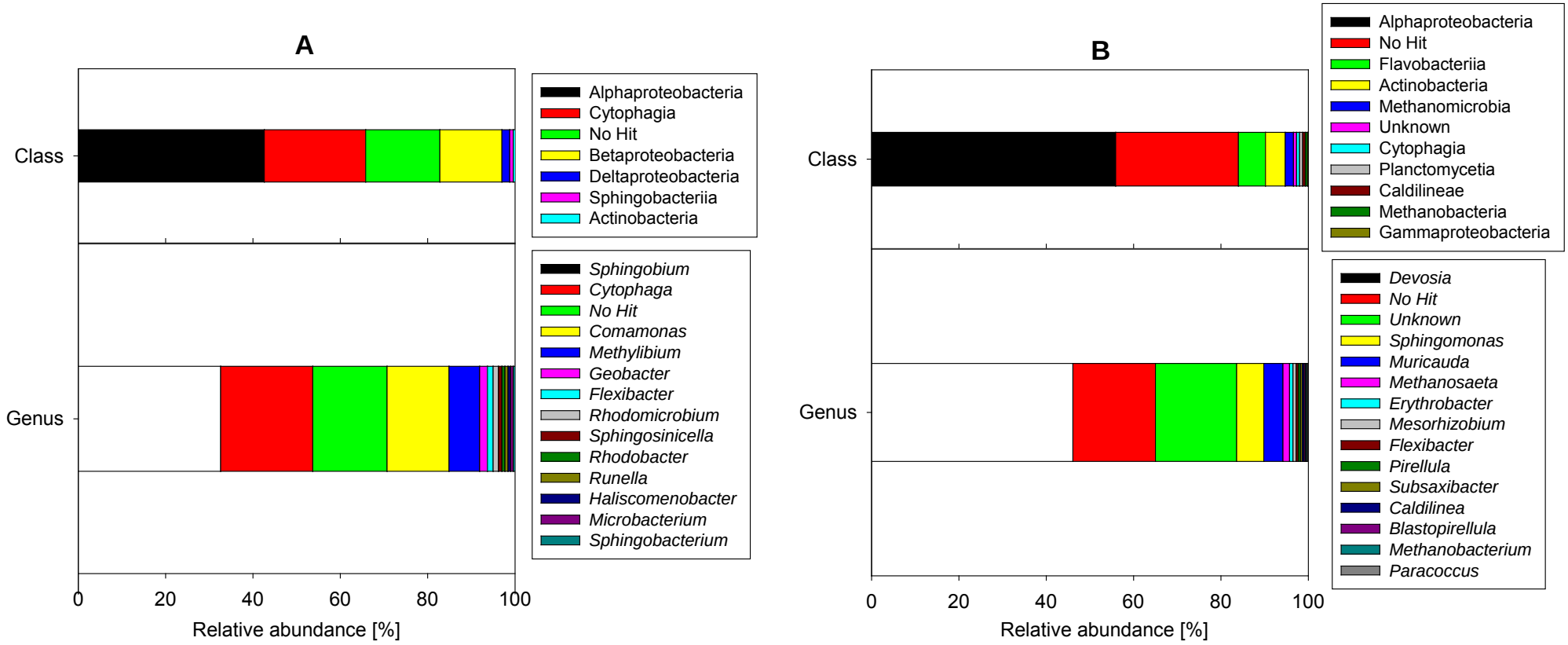
A
Class
Genus
0
20
40
60
80
100
Relative abundance [%]
Alphaproteobacteria
Cytophagia
No Hit
Betaproteobacteria
Deltaproteobacteria
Sphingobacteriia
Actinobacteria
Sphingobium
Cytophaga
No Hit
Comamonas
Methylibium
Geobacter
Flexibacter
Rhodomicrobium
Sphingosinicella
Rhodobacter
Runella
Haliscomenobacter
Microbacterium
Sphingobacterium
B
Class
Genus
0
20
40
60
80
100
Relative abundance [%]
Alphaproteobacteria
No Hit
Flavobacteriia
Actinobacteria
Methanomicrobia
Unknown
Cytophagia
Planctomycetia
Caldilineae
Methanobacteria
Gammaproteobacteria
Devosia
No Hit
Unknown
Sphingomonas
Muricauda
Methanosaeta
Erythrobacter
Mesorhizobium
Flexibacter
Pirellula
Subsaxibacter
Caldilinea
Blastopirellula
Methanobacterium
Paracoccus

Figure 4