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TREATMENT OF COMPLEX INDUSTRIAL WASTEWATERS CONTAINING AMMONIUM AND PHENOLIC COMPOUNDS USING GRANULAR SLUDGE IN CONTINUOUS AIRLIFT REACTORS

PhD Thesis

Supervised by

Julio Pérez Cañestro and María Eugenia Suárez-Ojeda

ZULKIFLY BIN JEMAAT

Bellatera – April 2013

JULIO PÉREZ CAÑESTRO, professor agregats, i MARÍE EUGENIA SUÁREZ-OJEDA, professora lector doctor, del Departament d'Enginyeria Química de la Universitat Autònoma de Barcelona,

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Dr. Julio Pérez Cañestro Dr. María Eugenia Suárez-Ojeda

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Summary

The simultaneous nitritation and phenolic compounds removal using aerobic granular reactors in continuous mode were studied in this Ph.D. thesis. The study is divided into two main subjects; the first one is devoted to the modeling of nitritation while the other part is dedicated to the experimental work of simultaneous nitritation and phenolic compounds removal using granular reactors.

In the modeling study, a mathematical biofilm model was developed to describe nitritation in aerobic granular reactors operating in continuous mode. The model incorporated a [DO]/[TAN] ratio control strategy to maintain the proportion between the concentrations of dissolved oxygen (DO) and total ammonia nitrogen (TAN) in the reactor effluent to a desired value. The model was validated with a large set of experimental results previously reported in the literature, as well as, data gathered from laboratory scale and pilot plant granular reactors treating reject water. The model was used to study the effect of: a) DO and TAN setpoints, b) operating temperature, c) biofilm characteristics (granules size, density) and d) ammonium concentrations in the influent on the achievement of full nitritation. The results indicated that full nitritation was stably maintained and enhanced by applying the [DO]/[TAN] ratio control strategy in the operation of aerobic granular sludge reactor. Moreover, the model predicted that aerobic granules size larger than 1.5 mm and high ammonium concentrations in the influent enhanced the achievement of stable full nitritation, while poor influence of the biofilm density was found with the simulation study. Furthermore, at low temperature, full nitritation with granular reactors was demonstrated to be possible.

In the experimental work, an airlift reactor was employed. In the reactor start-up, granular sludge from a reactor performing biological nutrient removal was used as inoculum. A synthetic wastewater containing high-strength ammonium concentrations (950 ± 25 mg N L^{-1}) was fed into the airlift reactor. The reactor was operated until partial nitritation was obtained. Once partial nitritation was achieved, the airlift reactor was bioaugmented with *p*-nitrophenol (PNP)-degrading activated sludge to enhance the growth of phenolic-degraders over the nitrifying granules. Immediately, *o*-cresol (up to 100 mg L^{-1}) or PNP (up to 15 mg L^{-1}) were progressively added to the high-strength ammonium influent and fed into the reactor with the objective of studying the simultaneous partial nitritation and *o*-cresol removal and the simultaneous nitritation and (PNP) removal.

First, in the study of simultaneous partial nitritation and *o*-cresol removal, a stably partial nitritation process was maintained for more than 100 days of operation. Moreover, full biodegradation of *o*-cresol was achieved during the whole experimental period. Also, *o*-cresol shock load events were applied and the partial nitritation process was kept stable and unaffected during these events. The achievable nitrogen loading rate (NLR_v) and *o*-cresol loading rate (CLR_v) were ca. 1.1 g N $L^{-1}d^{-1}$ and 0.11 g *o*-cresol $L^{-1}d^{-1}$, respectively. Analysis of fluorescent in-situ hybridization (FISH) indicated that *Acinetobacter* genus, betaproteobacterial ammonia-oxidizing bacteria and *Nitrobacter* sp. were present into the granules. Later, the operation of the reactor was continued, and an experiment devoted to the performance of the reactor under three sequentially alternating pollutant (SAP) scenarios was executed. In each one of the SAP scenarios, 15 mg L^{-1} of the secondary phenolic compounds (i.e. *p*-nitrophenol (PNP), phenol and 2-chlorophenol (2CP)) were added in the regular influent for a short period of time (between 20 to 25 days). The results illustrated that partial nitritation and *o*-cresol biodegradation were maintained without exhibiting any sign of inhibition by the presence of PNP or phenol. However, when 2CP was present in the influent,

90 % of the partial nitritation and 25 % of the *o*-cresol degradation was inhibited within three days. This finding suggests that the ammonia oxidizing bacteria (AOB) is more sensitive to 2CP inhibition than heterotrophs (*o*-cresol-degraders).

Second, in the study of simultaneous nitritation and PNP removal, nitritation was maintained during most of the operation period producing an effluent suitable for heterotrophic denitrification. However, in the first 175 days, PNP biodegradation was unstable and several accumulation episodes occurred. The oxygen limiting condition was found to be the main explanation of these events. The increase of dissolved oxygen concentration (DO) in the reactor from 2 to 4 mg O_2 L^{-1} permitted to achieve complete and stable PNP removal till the end of the experimental period. The achieved NLR_v and PNP loading rate ($PNP\text{-}LR_v$) were ca. 1.0 g N $L^{-1}d^{-1}$ and 16 mg PNP $L^{-1}d^{-1}$, respectively. Besides, the performance of the reactor was further assessed by performing two starvation studies, i) PNP starvation and ii) total starvation period (reactor shutdown). Results show that full recovery of PNP degradation was achieved within 2 days after the PNP starvation period ended, while full recovery of simultaneous nitritation and PNP removal was accomplished in just 11 days after the restart of the reactor.

In conclusion, the use of continuous aerobic granular reactors for the simultaneous nitritation and phenolic compounds removal is feasible. This could be regarded as a best available technique for the treatment of complex industrial wastewaters containing high-strength ammonium concentrations and phenolic compounds. Aerobic granules are proven to be resistant and resilient to the shock loads, to the alternating presence of recalcitrant compounds and to starvation periods; conditions frequently found in industrial wastewater treatment plants due to changes on the industrial production schedules. In the near future, we propose the simultaneous nitritation and phenolic compounds removal should be combined with either heterotrophic denitrification or Anammox for sustainable nitrogen removal.

Resumen

Esta tesis doctoral versa sobre la eliminación simultánea de compuestos fenólicos con reactores de biomasa granular trabajando en continuo. El estudio está dividido en dos partes principales; el primer tema trata sobre la modelización de la nitritación, mientras que el otro está dedicado al trabajo experimental sobre la nitritación y eliminación simultánea de compuestos fenólicos.

En el estudio de modelización, se desarrolló un modelo matemático de biopelícula para describir la nitritación en reactores de biomasa granular aerobia operando en continuo. El modelo incorpora una estrategia de control del ratio [DO]/[TAN], para mantener un valor deseado de la relación entre las concentraciones de oxígeno disuelto (DO) en el efluente del reactor y nitrógeno amoniacal total [TAN]. El modelo se validó con un gran número de datos experimentales previamente publicados en la bibliografía, así como con datos obtenidos de reactores granulares tratando agua de rechazo a escala laboratorio y piloto. El modelo se utilizó para estudiar el efecto de: a) las consignas de DO y TAN, b) la temperatura de operación, c) las características de la biopelícula (tamaño de partícula, densidad) y d) la concentración de amonio en el afluente, sobre la consecución de la nitritación completa. Los resultados indicaron que la nitritación completa se mantuvo estable y se potenció usando la estrategia de control de la proporción [DO]/[TAN] en la operación del reactor de biomasa granular aerobia. Además, el modelo predijo que gránulos aerobios mayores a 1.5 mm y concentraciones altas de amonio en el afluente potenciaba la obtención de nitritación completa estable, mientras que la densidad de biopelículas tenía poca influencia en este estudio. Además se demostró que era posible la nitritación total a bajas temperaturas con reactores de biomasa granular.

Para el trabajo experimental, se utilizó un reactor tipo *airlift*. Para la puesta en marcha del reactor, se utilizó como inóculo biomasa de un reactor de biomasa granular aerobia que realizaba eliminación de nutrientes. Como alimento del reactor se utilizó un agua residual sintética con un alto contenido de amonio (950 ± 25 mg N L^{-1}). El reactor se operó hasta la obtención de nitritación parcial. Una vez obtenida la nitritación parcial, el reactor se bioaumentó con un lodo activo que contenía biomasa degradadora de *p*-nitrofenol (PNP) para mejorar el crecimiento de microorganismos degradadores de fenol sobre los gránulos nitrificantes. Acto seguido, mientras el reactor trataba una carga elevada de amonio, se añadieron progresivamente al afluente *o*-cresol (hasta 100 mg L^{-1}) o PNP (hasta 15 mg L^{-1}), siendo éstos alimentados al reactor con el objetivo de estudiar la nitritación parcial simultánea a la eliminación de *o*-cresol o de PNP.

En el estudio de la nitritación parcial simultánea a la eliminación de *o*-cresol, se mantuvo el proceso de nitritación parcial estable durante más de 100 días de operación. Además, se obtuvo una biodegradación completa de *o*-cresol durante todo el periodo experimental. También se realizaron choques de carga de *o*-cresol, durante los cuales el proceso de nitritación parcial se mantuvo estable y sin verse afectado por esos eventos. Las cargas volumétricas obtenidas de nitrógeno (NLR_V) y de *o*-cresol (CLR_V) fueron de 1.1 g N L^{-1} d^{-1} y 0.11 g *o*-cresol L^{-1} d^{-1}, respectivamente. El análisis de hibridación in situ de fluorescencia (FISH) indicó que en los gránulos había presencia del género *Acinetobacter*, de bacterias amonio-oxidantes betaproteobacteriales y de *Nitrobacter sp*. Posteriormente, se continuó con la operación del reactor, y se llevó a cabo un experimento relacionado con el funcionamiento del reactor bajo tres escenarios de

alternancia secuencial de contaminantes (SAP). En cada uno de los escenarios SAP se añadieron 15 mg L^{-1} de compuestos fenólicos secundarios (i.e. PNP, fenol y 2-clorofenol (2CP)) al afluente por un periodo de tiempo corto (entre 20 y 25 años). Los resultados ilustraron que se mantuvo la nitritación parcial y la biodegradación de o-cresol sin mostrar ningún signo de inhibición por la presencia de PNP o de fenol. Sin embargo, en presencia de 2CP en el afluente, se registró durante tres días un 90% de la nitritación parcial y un 25% de la degradación de *o*-cresol. Estos resultados sugieren que las bacterias amonio oxidantes (AOB) son mas sensibles a la inhibición por 2CP que las heterótrofas (degradadoras de *o*-cresol).

En el estudio de la nitritación simultánea a la eliminación de PNP, se mantuvo la nitritación durante la mayor parte del periodo operacional, obteniéndose un efluente adecuado para la desnitrificación heterotrófica. Sin embargo, durante los primeros 175 días, la biodegradación de PNP fue inestable, observándose diversos episodios de acumulación de PNP. Esta acumulación se determinó que era debida a las condiciones limitantes de DO. El incremento de la concentración de DO en el reactor de 2 a 4 mg O_2 L^{-1} permitió obtener eliminación completa y estable de PNP hasta el fin del periodo experimental. Las NLR_V y la carga de PNP obtenidas fueron de 1.0 g N L^{-1} d^{-1} y 16 mg PNP L^{-1} d^{-1}, respectivamente. Además, se evaluó el funcionamiento del reactor realizando dos estudios de hambruna, i) hambruna de PNP y ii) hambruna total (parada del reactor). Los resultados mostraron que 2 días después al fin del periodo de hambruna se obtuvo una recuperación total de la degradación de PNP, mientas que la recuperación total de la nitritación simultánea a la eliminación de PNP se consiguió solo 11 después de volver a poner en marcha el reactor.

En conclusión, el uso de reactores de biomasa granular aerobia para realizar nitritación simultánea a la eliminación de compuestos fenólicos es factible. Ésta podría ser considerada la mejor técnica disponible para el tratamiento aguas residuales industriales complejas con contenido de amonio en alta carga y compuestos fenólicos. Se ha probado que la biomasa granular aerobia es resistente a sobrecargas puntuales, a presencia alterna de compuestos recalcitrantes y a periodos de hambruna; estas condiciones, debido a los cambios de planificación de la producción, pueden encontrarse frecuentemente en plantas de tratamiento de aguas residuales industriales. En un futuro próximo, proponemos que la nitritación simultánea a la eliminación de compuestos fenólicos podría combinarse tanto con la desnitrificación heterotrófica o con el proceso anammox para una eliminación sostenible del nitrógeno.

Thesis outline

The thesis is divided into four main parts. The main content of each part and the corresponding chapters will be detailed as follows.

Part I (General Introduction) consists of three chapters i.e. Chapters 1, 2 and 3. In Chapter 1, an overview of the state of the art on the performed studies in the field of biological nitrogen and phenolic compounds removal from industrial wastewaters is presented. Special emphasis was paid to ammonium oxidation by the 'nitrite route' i.e. partial nitritation and to aerobic biodegradation of phenolic compounds. The aerobic granular sludge technology was also highlighted in particular for the treatment of nitrogen and phenolic compounds. In Chapter 2, the main goal and objectives of the PhD study are pointed out and elaborated. Finally, in Chapter 3, the general materials and methods used in the whole experimental study are described. However, specific information regarding materials and methods corresponding to each one of the experiments performed are described in detail in each the corresponding chapters.

In Part II (Modeling of Nitritation in Granular Reactors), Chapter 4 and 5 are presented. In Chapter 4, a mathematical biofilm model was developed to describe nitritation using an aerobic granular sludge reactor operating in continuous mode. The implementation of [DO]/[TAN] ratio control tested in a real application of a continuous reactor of aerobic granular sludge was incorporated into the model and simulated. Several scenarios were assessed to investigate the stability and robustness of the applied control strategy. The model was validated with experimental results from pilot plant nitrifying granular reactors treating a high-strength-ammonium concentration wastewater. Furthermore, the model was used to study the achievement of full nitritation in front of changes in DO and TAN setpoints, operating temperature and the granule size. Additionally, the importance of controlling the TAN concentration was highlighted with different scenarios, in which periodic disturbances were applied mimicking a poor control situation. The findings of the modeling study are discussed in Chapter 4. In Chapter 5, the previous developed model was further explored for the extension study on the effect of ammonium concentrations in the influent and biomass density on the achievement of full nitritation using aerobic granular sludge reactors. The model was validated with experimental results from lab-scale and pilot plant nitrifying granular reactors treating high-strength-ammonium concentration wastewaters. The results of the modeling study are discussed in detail, in Chapter 5. Prior to the experimental study in Part III, the developed model was utilised in the pre-feasibility study of simultaneous nitritation and phenolic compounds removal. Several scenarios were designed and simulated to determine the granular reactor start-up for simultaneous nitritation and phenolic compounds removal.

In Part III (Simultaneous Nitritation and Phenolic Compounds Removal), Chapter 6, 7, 8 and 9 are presented. The feasibility study of simultaneous partial nitritation and *o*-cresol removal using a continuous reactor of aerobic granular sludge was performed, and the findings are discussed in Chapter 6. In addition, the performance and stability of the reactor treating ammonium and *o*-cresol experiencing two shock load events were also assessed and discussed. In Chapter 7, the aerobic granular reactor performing simultaneous partial nitritation and *o*-cresol removal was experienced several SAP scenarios. In each of the SAP scenario, a secondary phenolic compound was introduced in the influent for a period of 3 weeks. The selected secondary phenolic compounds utilized were phenol, PNP and 2-chlorophenol. The results and the findings are discussed in detail, in Chapter 8. In Chapter 8,

the feasibility study of simultaneous nitritation and PNP removal using an aerobic granular sludge reactor operating in continuous mode was performed, and the results are discussed in detail in this chapter. The experimental work in Chapter 8 is further extended in Chapter 9, which the aerobic granular reactor performing simultaneous nitritation and PNP removal was submitted to starvation periods. Two starvation scenarios were performed; i) PNP starvation and ii) PNP and TAN total starvation, i.e. reactor shut-down. The subsequent reactivation of biological processes was evaluated and discussed in this chapter.

Finally, Part VI (Conclusions) gives an overview of the main achievements of this thesis, and points out the topics for future research derived from this study. Figure 1 illustrates the general outline of the thesis through a graphical abstract.

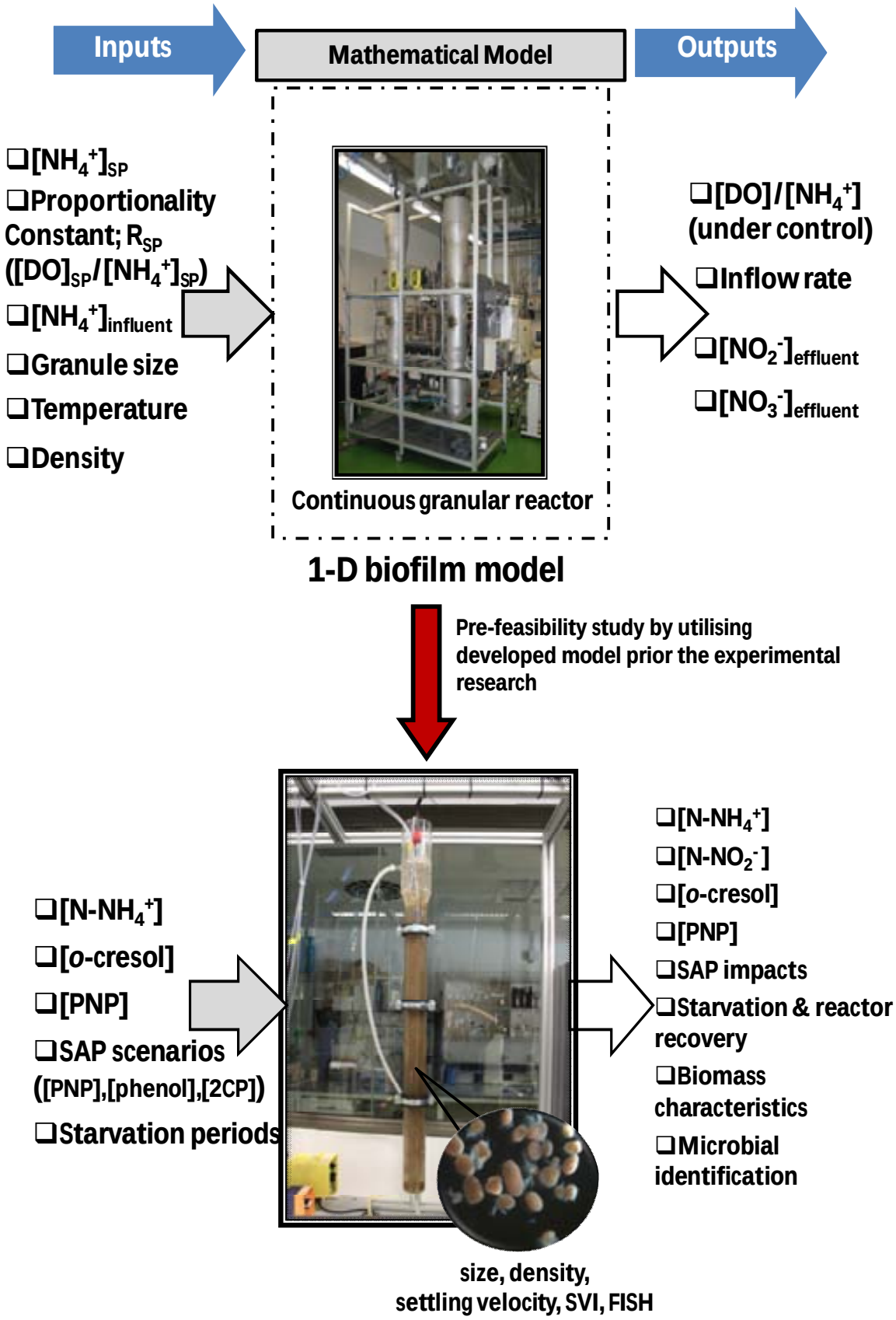

Figure 1 General overview of the thesis outline through a graphical abstract commencing from mathematical modeling of nitritation and subsequent experimental research.

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PART I

GENERAL INTRODUCTION

Chapter 1

Introduction

Summary

In this chapter, an overview of the state of the art with regard to industrial wastewaters, the challenges in their treatment and the applicable legislation is presented focussing the discussion on certain industries producing wastewaters containing ammonium and phenolic compounds. A detailed literature review related to the biological treatment of nitrogen and phenolic compounds either using activated sludge or aerobic granular systems was performed. The main achievements reported in the literature have been summarized and depicted in table format. Finally, a brief discussion on the future perspectives on the simultaneous removal using aerobic granular sludge technology is highlighted.

1.1 Industrial wastewater characteristics, difficulties and regulations

It is known that industrial sectors are vital for the world economy. Industries are spurring and becoming the backbone of the most developed countries' economy for instance Japan, Germany and United States. However, do you ever realise that the industrial sector, besides producing useful products, it is certainly generating a significant portion of the wastewater discharged worldwide? Direct discharge of industrial wastewaters into rivers, lakes and coastal areas has resulted in serious pollution problems in the environment, and is causing negative effects in various ecosystems on which human life relies on. There are many types of industrial wastewater, each industrial sector generates its own particular combination of pollutants. The amount of wastewater generated depends on the technical level of the process in each industrial sector and could be gradually reduced with the improvement of industrial technologies. The increasing rates of industrial wastewater in developing countries are thought to be much higher than those in developed countries, in fact it has been predicted that, in the early 21st century, the industrial wastewater pollution problem will shift from the developed countries to the developing countries (Shi, 2009). Therefore, the industrial pollution problems faced by different countries worldwide are different.

In developed countries, the environmental pressure created by traditional industrial activities i.e. the emission of pollutants from iron and steel, metal fabrication, mining activities, textile industries and petrochemicals has grown slowly in recent decades, but other types of environmental problems have received growing attention, e.g. contamination of soil and large volumes of waste material that are often dumped in the immediate surroundings of the factories, with subsequent high costs for remedial treatments (Wen, 2009). Started in the 1980s, the efforts of many countries of Organization for Economic Cooperation and Development (OECD) to reduce pollution became apparent and many environmental problems have been solved since then. These experiences have favour environmental protection and encourage research and development, thereby promoting new technologies in the industry to further minimize environmental risks. It also provides the required financial conditions under which large investments in new technology, necessary for further reducing environmental effects, can be made. As a result, the prerequisites are created for a sustainable industrial development with lower requirements for natural resources and enhanced waste minimization and recycling (Wen, 2009).

In developing countries, the environmental pressure coming from the traditional pollutants created by industries is still very heavy. Efforts have been made from many developing countries to set away areas called 'free industrial zones' in which the industrial plants are located in a specific designated land. These zones are regarded from perspectives of custom regulations, taxation, working hours, occupational safety and even environmental protection. However, by concentrating industries at the same site, the environmental pollution situations become more complex. Nevertheless, for both developed and developing countries, the growing technology-based industries created new problems due to the use of toxic material in their production processes, which can cause soil and water contamination. The wastewater generated from such industries is complex and hazardous in nature and need to be treated properly (Kumar et al., 2012). Often, these wastewaters are difficult to treat biologically. As such, the innovative treatment of industrial wastewater specifically designed for the particular type of effluent is critically required.

Typically, industrial wastewaters may contain high levels of inorganic pollutants, organic pollutants which can be easily biodegradable, but whose impact load on the ecosystems,

either in total suspended solids (TSS) or chemical oxygen demand (COD) may be in the tens of thousands mg L^{-1} (Ng, 2006). Industrial wastewaters also may contain recalcitrant and toxic compounds which are difficult to treat biologically. Table 1.1 presents typical industrial pollutants found in the wastewaters of certain industrial sectors. The COD and inorganic compounds are the major sources of pollutants in most of the industrial sector listed (Table 1.1). Focusing on the petrochemical, coke and chemical industry, their wastewaters may be characterised by high concentration of organic, phenolic and ammonia compounds (Table 1.2). Common phenolic compounds found in such industrial wastewaters are phenol, nitrophenols, cresols and chlorinated phenols. Phenolic compounds are known to be toxic, carcinogenic and mutagenic to aquatic organisms at very low concentrations (EPA, 1994). Typically, the presence of phenolic compounds in wastewaters is characterised by the total phenols concentration. If these compounds enter to wastewater treatment facility, especially in the case of the biological treatment unit and an appropriate monitoring is not in place, they could inhibit the existing biological processes. Moreover, ammonia compound in industrial wastewaters may cause serious environmental problems if this compound is not properly eliminated before discharge into the receiving water bodies. A too high nitrogen concentration in the receiving waters can lead to eutrophication, hypoxia and loss of biodiversity and habitat (Galloway et al., 2003, 2008).

Table 1.1 Typical industrial wastewater pollutant characteristics, sorted by industrial sectors (Shi, 2009)

Sectors	**Pollutants**
Iron and steel	COD, oil, metals, acids, phenols, cyanides
Textiles and leather	COD, solids, sulphides, chromium, ammonia, dyes
Pulp and paper	COD, solids, chlorinated organic compounds, phenols
Petrochemicals and refineries	COD, mineral oils, phenols, ammonia, chromium, aromatics
Chemicals	COD, organic chemicals, heavy metals, suspended solids, cyanides, phenols, aromatics, ammonia
Non-ferrous metals	Fluorine, suspended solids
High-technology (e.g: microelectronics, optoelectronics, semiconductor)	COD, ammonia, organic chemicals, suspended solids
Mining	Suspended solids, metals, acids, salts
Coke and gas	COD, organic chemicals, ammonia, phenols, cyanides
Pharmaceutical	COD, organic chemicals, aromatics, ammonia

At present, wastewaters from petrochemical, coke and chemical industry are treated by a combination of physicochemical and biological processes. Figure 1.1 illustrates the general schemes of wastewater treatment plants in these industries. The typical wastewater treatment plants includes: i) physical processes for the removal of oil, grease and heavy hydrocarbons ii) physicochemical processes for the removal of macromolecular and colloidal substances and iii) biological wastewater processes aiming to remove organics, nutrients and particularly short-chain hydrocarbons (Tobiszewski et al., 2012). In the biological processes, systems like activated sludge, membrane bioreactor, aerated lagoon, trickling filters, nitrification or/and denitrification are most widely used. In certain cases, the effluent after biological processes is sent to tertiary treatment (sand filtration, chemical oxidation) for post-treatment prior to discharge or even for reusing purposes. The current treatment processes (combined physicochemical and biological treatment) have few drawbacks associated, for instance high energy demand, high operating costs, problems with by-products disposal and most importantly; these methods cannot sufficiently remove several of the toxic and recalcitrant contaminants. In a biological treatment involving nitrification and denitrification processes, where ammonia is oxidized and removed as nitrogen gas, these processes could be inhibited and ineffective, if recalcitrant compounds enter into the system, consequently, the effluent quality deteriorates significantly.

Table 1.2 Typical compounds present in wastewaters from petrochemicals and refineries, chemicals and coke and gas sector. Chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS).

Sectors	Compound concentration (g L^{-1})					Reference
	COD	BOD	Ammonia	Phenols	TSS	
Petrochemicals and refineries	0.07 -1.0	0.01-0.36	0.02 – 0.07	0.01-0.2	0.02-0.6	(Diya'uddeen et al., 2011)
Chemicals	9.6 – 17	1.4 - 4.3	0.6 – 1.7	0 – 5	6 – 10.3	(Abeliovich, 1985; UNEP, 1998)
Coke and gas	0.9-3.1	0.5-1.4	0.5-2.2	0.01 -0.5	0.02-3.3	(Chang et al., 2008)

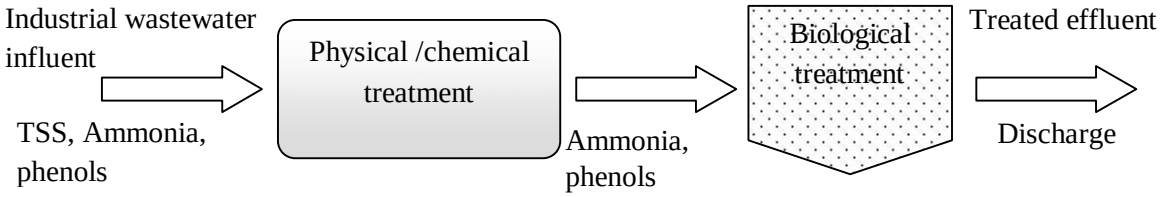

Figure 1.1 A general treatment scheme for industrial wastewater containing ammonia and phenols in certain industry.

On top of that, several difficulties or operational changes encountered by industries during the production and processing activities could cause the biological treatment methods of

industrial wastewater treatment plants to be uncertain and ineffective (Sipma et al., 2010). Such difficulties faced by the biological treatment due to the instability of industrial activities are:

i) Operational problems, caused by pH, temperature, salinity changes
ii) High organic loads, shock loads and toxic loads
iii) Presence of sequentially alternating pollutants (SAP) due to a variable production schedule
iv) Long-term starvation periods due to maintenance or minor upgrading purposes
v) Presence of toxic compounds due to the change of process route or acquiring new technology to meet customers' demand and requirements

Often, fluctuation in wastewater characteristics can be dampened by the provision of a flow equalization or buffer tank prior to the biological process (Sipma et al., 2010). In many cases, WWTPs are normally run at a more constant flow rate because of such storage reservoirs provided in the system (Droste, 1997). Although flow equalization is determined based on hydraulics rather than concentrations, it often prevents biological downstream processes from shock loads or high concentrations of toxic chemicals and may further provide in-system for pH neutralization, minimizing the consumption of chemicals (Tchobanoglous et al., 2004). However, highly variable concentrations of different components will make it impossible to achieve a uniform loading for all components (Droste, 1997). Because of such variability, flow equalization is likely of limited effectiveness. Moreover, weekly or monthly alternated production schemes that generate distinct wastewater characteristics, and for which flow equalization is ineffective, ensures the need for a robust treatment processes capable to cope with considerable fluctuations (Sipma et al., 2010).

Besides, the industries also need to ensure that the treated effluent is meeting the standards for water discharge set by the government or local authorities before it can be released to the receiving body. Nowadays, a stricter legislation governing the discharge of effluents has been imposed by many counties, aiming to ensure that our environment is well protected and uncontaminated. Several examples of legislation of water quality for discharge imposed to the industrial sectors are described in Table 1.3. European Commission (EC) has established a guideline under the urban wastewater treatment directive 91/271/EEC concerning to the collection, the treatment and the discharge of urban wastewater and to the treatment and discharge of wastewater from certain industrial sectors (EEC, 1991). Each member of the European Union is required to adapt, implement and bring into force the laws, regulations and administrative provisions necessary to comply with this directive. Although phenols are not prescribed as one of the core parameters in this directive, phenolic compounds have to be closely monitored and frequently reported to the EC under provisions of Directive 2008/1/EC concerning to the integrated pollution prevention and control. Another example, the Spanish Government through RD 849/1986 imposed a limit below 50 mg N L^{-1} for ammonia and between 1.0 to 0.5 mg L^{-1} for phenols in industrial wastewater discharges. Moreover, in Spain, autonomous communities and even municipalities could also establish its own laws and regulations regarding discharge limits. Catalunya, for instance, has established a list of parameters for the discharge of industrial wastewater to the public sewage under Decret 130/2003. According to the Decret 130/2003, the effluent discharge limit for nitrogen ammonium and phenols is 60 and 2 mg L^{-1}, respectively. As an example of a South East Asian Country, the Malaysian Government has enforced a standard limit below 20 mg N L^{-1} for ammonia and 1 mg L^{-1} for phenols for industrial effluent quality under the Environmental Quality Regulations 2009. In conclusion, this indicates that the stricter regulations and laws have been enforced worldwide regarding the standards for wastewater discharge limits that

must be fulfilled by industries before the effluent can be discharged into receiving bodies. It also illustrates the efforts and concerns of most of the countries in the world regarding the environmental issues for sustaining a healthy environment.

Table 1.3 Water quality legislations in Spain, Catalunya and Malaysia.

Legislation	Major compound discharge limit (mg L^{-1})					Reference
	COD	BOD	Ammonia	Phenols	SS	
RD 849/1986	160-500	28-210	15-50	0.5-1.0	80-300	(Spanish Government, 1986)
Decret 130/2003	1500	750	60[a]	2	750	(Generalitat de Catalunya, 2003)
Environmental Quality (Industrial Effluent) Regulations 2009	N/A	20-50	10-20	0.001-1.0	50-100	(DOE, 2009, Malaysia)

[a]the limit is set for NH_4^+

Therefore, the need to adopt advanced treatment processes, including aerobic granular sludge are crucial either to ensure the quality standards for discharged water abide by legislation or to overcome the difficulties faced by industries on the current treatment methods employed.

The application of aerobic granular sludge to treat various types of industrial wastewaters including petrochemical and chemical industry is proven feasible and practical (Adav et al., 2009; Maszenan et al., 2011). Compared to conventional activated sludge systems, the granular biomass could retain a higher biomass concentration in the reactor and avoid biomass washout occurrence due to its excellent settleability, compact and dense microbial structure (Gao et al., 2011a). Moreover, a diversified microbial aggregate within the granule structure provides a platform for simultaneous removal of several contaminants (Adav et al., 2008). Granular biomass are also resilient to toxic compounds, shock loads events and its biological processes could be reactivated and recovered easily after a long-term starvation periods (Gao et al., 2011a). Above all, the application of aerobic granular sludge to treat complex industrial wastewaters has been demonstrated to be useful and promising in the wastewater treatment. Nevertheless, further research is needed to explore the possibility of using the same granular biomass for degrading different recalcitrant compounds, including its ability to withstand in front of instabilities of the upstream process (SAP events and long-term starvation periods). The efforts will fill the gaps relating to the application of aerobic granular technology, so that it will proliferate completely, providing the opportunity to deal with water pollution and sustain a healthy environment.

1.2 Biological wastewater treatment

The overall objective of the biological treatment of industrial wastewater is to remove or reduce the concentration of organic and inorganic compounds. The organic compounds include proteins, carbohydrates, oils, fats and synthetic organic molecules such alkenes, aromatics, alcohols and volatile organic compounds. Meanwhile, the inorganic compounds

include ammonia, nitrite, nitrate, sulphur and phosphorus (Tchobanoglous et al., 2004). The removal of soluble COD and the stabilization of organic matter found in wastewater are accomplished biologically using a variety of microorganisms, principally bacteria (Tchobanoglous et al., 2004). Microorganisms are used to oxidise the dissolved and particulate carbonaceous organic matter into simple end products and additional biomass (Figure 1.2). Microorganisms are also used to remove nitrogen and phosphorus in wastewater treatment processes. Specific bacteria are capable of oxidizing ammonium to nitrite and nitrate (nitrification), while other bacteria can reduce the oxidized nitrogen to gaseous nitrogen. For phosphorus removal, biological processes are configured to encourage the growth of bacteria with the ability to take up and store large amounts of inorganic phosphorus and subsequent solids separation.

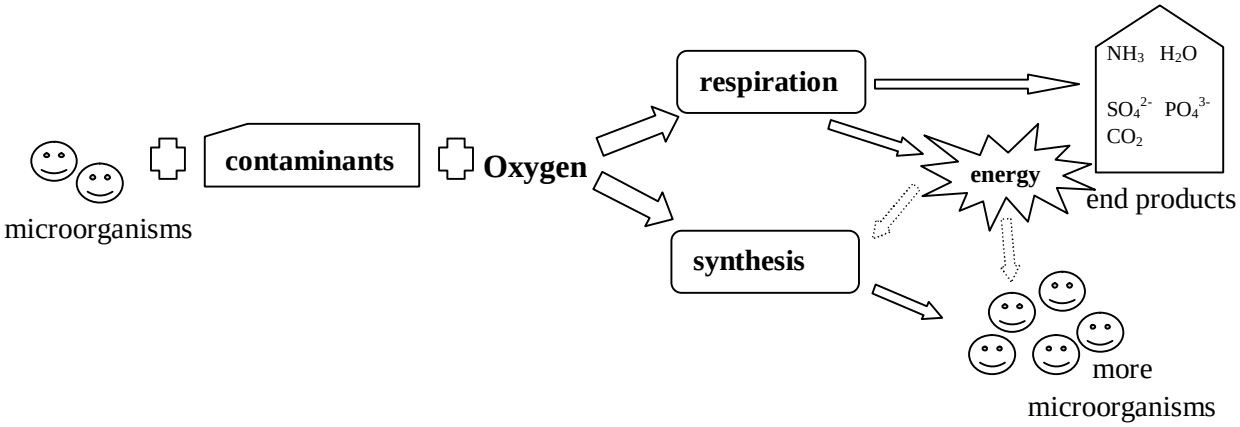

Figure 1.2 Aerobic oxidation of contaminants dissolved in wastewater by microorganisms and their products produced. Adapted from Tchobanoglous et al. (2004).

1.2.1 Biological nitrogen removal

The nitrogen compound most frequently found in wastewaters is ammonium (NH_4^+) which can be removed by physicochemical or biological processes. Because biological nitrogen removal (BNR) is more effective and relatively inexpensive, it has been widely adopted in favour of the physicochemical processes (Ahn, 2006). The complete biological nitrogen removal can be accomplished either in an aerobic or anaerobic condition. The conventional BNR, using the nitrification and denitrification pathway proceeds slowly due to low microbial activity and yield. The process is generally performed on urban wastewater containing low nitrogen concentration. In the past few years, several novel and cost-effective biological nitrogen removal processes have been successfully developed. Such novel processes including partial nitritation, aerobic denitrification, anaerobic ammonium oxidation (Anammox), and its combined system (for example, completely autotrophic nitrogen removal over nitrite, CANON). The biological nitrogen cycle is complex and plays an important role in the environment. This cycle is recently re-classified according to potential biochemical pathway as shown in Figure 1.3. Besides, the ammonium concentration in wastewater, the COD/N ratio also plays an important role for the selection of suitable biological nitrogen removal (Vázquez-Padín, 2009). For instance, in the case of COD/N > 20, the assimilation of nitrogen by heterotrophic bacteria is sufficient to remove nitrogen. In the case of 20 < COD/N < 5, the removal of nitrogen can be carried out by assimilation and nitrification-denitrification pathway. Finally, for wastewater containing COD/N < 5 ratio, the nitrogen removal by nitrite route processes is preferred (partial nitritation-denitrification, or partial nitritation-Anammox). In this sense, a combined system for nitrogen removal based on partial

nitritation with Anammox has various advantages, such as no need for external carbon addition, negligible sludge production and less energy and oxygen requirements than the conventional process (Jetten et al., 2002). The ammonium oxidation from wastewater containing high-strength ammonium concentrations by partial nitritation will be the aim of the thesis.

Figure 1.3 Biological transformations in the nitrogen cycle. Adapted from van Benthum (1998).

Nitrification

Nitrification is the biological oxidation of ammonia into nitrite followed by oxidation of nitrite into nitrate through the action of two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). The nitrification process is carried out in two sequential stages: the ammonia oxidation to nitrite (nitritation) and the subsequent oxidation of nitrite to nitrate (nitratation). It should be noted that the two groups of autotrophic bacteria are distinctly different. Starting with classical experiments on nitrification by Winogradsky (1891), the bacteria genera commonly noted for nitrification in wastewater treatment are the autotrophic bacteria *Nitrosomonas* and *Nitrobacter*, which oxidize ammonia to nitrite and then to nitrate, respectively. Other autotrophic bacteria genera capable of obtaining energy from the oxidation of ammonia to nitrite are *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* (Painter, 1970). Besides *Nitrobacter*, nitrite can also be oxidized by other autotrophic bacteria as *Nitrococcus*, *Nitrospira*, *Nitrospina* and *Nitroeystis* (Wagner et al., 1995).

The energy-yielding two-step oxidation of ammonia to nitrate is as followed:

Nitritation: $$2NH_4^+ + 3O_2 \xrightarrow{AOB} 4H^+ + 2H_2O \quad \text{(Eq. 1.1)}$$

Nitratation: $$2NO_2^- + O_2 \xrightarrow{NOB} 2NO_3^- \quad \text{(Eq. 1.2)}$$

Total oxidation reaction: $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$ (Eq. 1.3)

Based on the above total oxidation reaction, the oxygen required for complete oxidation of ammonium is 4.57 g O_2/g N oxidized with 3.43 g O_2/g N used for nitrite production and 1.14

g O_2/g N oxidized. When synthesis is considered, the amount of oxygen required is less than 4.57 g O_2/g N. The following reaction steps are applied when the biomass growth and the bicarbonate used as carbon source are being considered in the oxidation.

$$55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7O_2N + NO_2^- + 104H_2CO_3 \quad \text{(Eq. 1.4)}$$

$$400NO_2^- + NH_4^+ + 195O_2 + HCO_3^- + 4H_2CO_3 \rightarrow C_5H_7O_2N + 400NO_3^- + 3H_2O \quad \text{(Eq. 1.5)}$$

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.021C_5H_7O_2N + 0.98NO_3^- + 1.88H_2CO_3 + 1.041H_2O \quad \text{(Eq. 1.6)}$$

Based on global bioreaction (Eq. 1.6), it will be noted that nitrification:

i) Requires 4.18 g O_2 for each g of ammonium (as N) oxidized
ii) 0.15 – 0.17 g of new cells are formed, where the bacteria composition is expressed as $C_5H_7O_2N$.
iii) In term of alkalinity, 8.92 g of bicarbonate is needed for each g of ammonium (as N). The quantity of inorganic carbon could be a limiting factor during nitrification.
iv) pH of the system tends to decrease due to alkalinity removal.

Maintaining nitrification would be relatively simple for WWTPs when operating in steady state. However, the desired steady state conditions often do not exist in the WWTP environment. For instance, all treatment plants experience significant fluctuation in daily flow, organic loading, nitrogen loading, temperature and concentrations of toxic chemicals. The nitrification process can be easily upset and lose efficiency with the numerous changes that could occur on a daily basis. In the worst case, the treatment facilities could violate its ammonium discharge limit. These upsets may last only a short period (a few days or weeks) or can be chronic problems. In general, several factors need to be monitored to ensure the successful of the nitrification process such as temperature, pH, dissolved oxygen, sufficient ammonia concentration, organic load, presence of toxic compounds, among others.

Denitrification

Denitrification process is the reduction of nitrate and nitrite to nitrogen gas in the absence of oxygen (anoxic conditions). Nitrite and nitrate act as electron acceptors. Biological denitrification is an integral part of the BNR, which involves both nitrification and denitrification. Bacteria capable of denitrification are both, heterotrophic and autotrophic. The heterotrophic organisms include the following genera: *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Moraxella*, *Pseudomonas*, *Rhizobium* among others (Payne, 1981). In addition, Gayle et al., (1989) listed *Halobacterium* and *Methanomonas*. *Pseudomonas* are the most common and widely distributed of all denitrifiers, and have been shown to use a wide array of organic compounds as carbon source including methanol, carbohydrates, organic acids, alcohols, benzoates, and other aromatic compounds (Payne, 1981). Most of these bacteria are facultative aerobic organisms with the ability of using oxygen, as well as, nitrate or nitrite, and some of them can also carry out fermentation in the absence of nitrate or oxygen. These groups of organisms grow heterotrophically if an organic carbon source is present (Gayle et al., 1989). At present, the heterotrophic denitrification is the most common and widely used in WWTPs

Autotrophic nitrifying bacteria, such as *Nitrosomonas europaea*, can use nitrite to oxidize ammonia, with the production of nitrogen gas, when dissolved oxygen is not present (Bock et al., 1995). With oxygen present, these bacteria oxidize the ammonia with oxygen as the electron acceptor. Ammonia oxidation with the reduction of nitrite under anaerobic conditions has been shown at temperatures above 20 °C in the Anammox process, which was discovered in the mid-1990s (Strous et al., 1997). The bacteria in Anammox process are different than the autotrophic nitrifying bacteria described above, in that it cannot use oxygen for ammonia oxidation (Jetten et al., 1999). The Anammox bacteria could not be isolated and grown in pure culture (Strous et al., 1999), but an enrichment was obtained by density purification for 16S rRNA extraction and analysis. Phylogenetic analysis showed that the bacteria is in the order *Planctomycetales*, a division with the domain Bacteria. Under anaerobic conditions the ammonia oxidation rate by the Anammox bacteria was shown to be 6 to 10 times faster than that for *N. Europaea* (Jetten et al., 1999). Recently, Anammox process for nitrogen removal from urban or industrial wastewaters has gained a tremendous research interest in the scientific community.

As explained before, the heterotrophic denitrification process requires the presence of a source of organic carbon as an electron donor and then, nitrate would acts as the last electron acceptor in the respiratory chain substituting the oxygen. The reduction is carried out by subsequent steps through different oxidation states of nitrogen and the global stoichiometry with acetic acid as organic carbon source (for example) is represented in the following reaction steps;

$$2CH_3COOH + 8NO_3^- \rightarrow 4CO_2 + 8NO_2^- + 4H_2O \quad \text{(Eq. 1.7)}$$

$$CH_3COOH + 8NO_2^- + 2H_2O \rightarrow 2CO_2 + 8NO + 8OH^- \quad \text{(Eq. 1.8)}$$

$$CH_3COOH + 8NO \rightarrow 2CO_2 + 4N_2O + 2H_2O \quad \text{(Eq. 1.9)}$$

$$CH_3COOH + 4N_2O \rightarrow 2CO_2 + 4N_2 + 2H_2O \quad \text{(Eq. 1.10)}$$

Total reaction: $$5CH_3COOH + 8NO_3^- \rightarrow 10CO_2 + 4N_2 + 6H_2O + 8OH^- \quad \text{(Eq. 1.11)}$$

Rewriting Eq. 1.11 taking into account the equilibrium of CO_2 gives Eq. 1.12.

$$NO_3^- + 0.625CH_3COOH \rightarrow HCO_3^- + 0.25CO_2 + 0.5N_2 + 0.75H_2O \quad \text{(Eq. 1.12)}$$

From the Eq. 1.12, it can be inferred that the denitrification process originates an increase in the medium alkalinity and that 40% of the organic matter needed is used to reduce nitrate to nitrite.

1.2.2 Advanced biological nitrogen removal

It is known that nitrification involves two sub-processes; oxidation of ammonia to nitrite and oxidation of nitrite to nitrate. In general, the presence of nitrite is undesired in wastewater treatment or other domains, however, some recent advanced BNR processes prefer nitrite as an intermediate (Sinha and Annachhatre, 2006). The so-called 'nitrite route' is a shortened nitrification process until nitrite, and then subsequent denitrification of the nitrite to nitrogen gas. The need of treatment for high-strength ammonium-concentration wastewater has motivated the research of new processes and optimal configurations. These novel treatments

i.e. partial nitrification, SHARON (Single reactor High activity Ammonia Removal over Nitrite), Anammox, CANON (Completely Autotrophic Nitrogen removal Over Nitrite), OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification), aerobic deamonification are briefly described in this section. The general principle of these processes is illustrated in Figure 1.4.

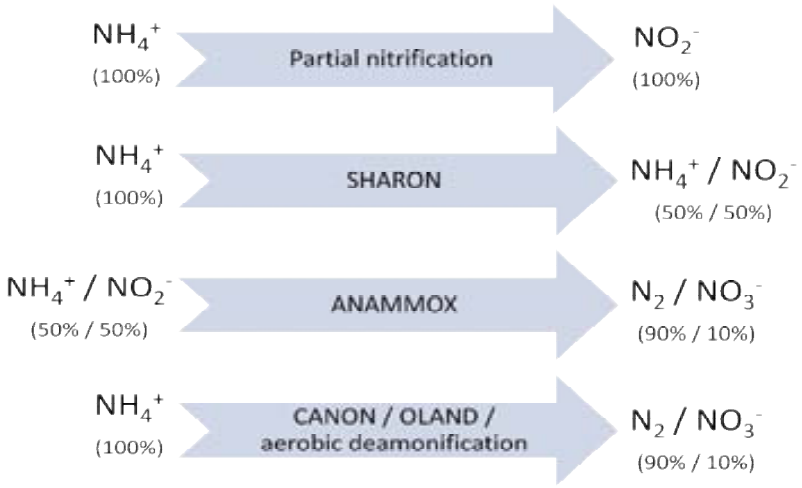

Figure 1.4 Schematic diagrams of partial nitrification, SHARON, Anammox, CANON, OLAND and aerobic deamonification processes. In brackets: percentages of nitrogen compounds in the flow (After Jubany, 2007).

Partial Nitrification

Partial nitrification, also known as nitritation is the oxidation of ammonium to nitrite by the AOB, while the oxidation of nitrite to nitrate carried out by NOB must be prevented. In this case, the further denitrification of the produced nitrite to nitrogen gas could be performed under autotrophic or heterotrophic conditions. Such combined biological nitrogen systems are partial nitrification-heterotrophic denitrification or partial nitrification-Anammox. In this way, several advantages with respect to the complete nitrification could be attained (Turk and Mavinic, 1987; Peng and Zhu, 2006): i) 40% reduction in COD requirements during denitrification; ii) 63% higher rate of denitrification; iii) 300% lower biomass production during anoxic growth; iv) 25% save of the oxygen requirements for nitrification due to suppression of the nitratation (oxidation of nitrite to nitrate) and v) 20% reduction of CO_2 emissions due to the denitrification from nitrite instead of nitrate. One example of successful partial nitrification system is the SHARON process developed in 1997 (Hellinga et al., 1998). This process is carried out in a conventional continuous stirrer tank reactor (CSTR) with suspended biomass and no sludge retention. At elevated temperature (30 and 40 °C) and low sludge retention time (SRT), AOB are selectively retained while the slow growing NOB, with growth rates lower than AOB at these conditions, are washed out from the system. Both nitrification and denitrification take place in the CSTR using intermittent aeration. Nitrogen removal efficiency close to 100% can be achieved.

The most critical condition that is needed for the success of partial nitrification process is to suppress nitrite oxidation without excessively retarding ammonium oxidation rate. Generation and maintenance of nitritation reactor requires that the NOB to be washed out from the system, or suppress suitable conditions to re-establish NOB growth (Sinha and Annachhatre, 2006). Unfortunately, AOB and NOB could be found almost everywhere and

therefore, it might be difficult to find conditions favouring one over the other (Egli et al., 2003). One powerful tool to achieve this condition is biochemical selection by inhibition of nitrite oxidation. Nitrite accumulation studies have been performed focused on several factors such as free ammonia (FA) and free nitrous acid (FNA) inhibition, pH, temperature and DO concentration (Turk and Mavinic, 1989a).

Several strategic parameters can be controlled to achieve partial nitrification to nitrite:

i) Temperature and SRT: At the usual operational temperatures of a WWTP no nitrite build-up is registered due to higher growth rate of NOB in the range of temperatures from 10 to 20 °C. However, the oxidation of ammonium has higher activation energy than the oxidation of nitrite. Therefore, operating a reactor above 25 °C would allow a selection of SRT for AOB and the washout of NOB which grow slower than AOB (Hellinga et al., 1998; van Loosdrecht and Salem, 2006). Raising temperature can, not only promote the growth rate of AOB, but also expand the differences of specific growth rates between AOB and NOB.

ii) Dissolved oxygen (DO): Operation at low DO level, between 0.7 and 1.4 mg L^{-1}, will effectively suppress the activity of NOB (Ruiz et al., 2003). AOB have lower oxygen affinity than NOB and the oxidation of nitrite to nitrate can be controlled by manipulating the DO concentration at low values. Nitrite accumulation was for instance, obtained by manipulating the DO concentration in biofilm systems (Garrido et al., 1997; Bernet et al., 2005). Several studies also had implemented a control strategy for the air flow rate to prevent nitratation (Guo et al., 2009a, 2009b; Yang et al., 2010). Jubany et al. (2009) demonstrated that by implementing local control loop for pH and DO (based on oxygen uptake rate (OUR)), a fast and stable NOB washout was achieved, thus a stable long-term operation of partial nitrification could be maintained. Nevertheless, using low DO concentration to produce nitrite could be disregarded because of simultaneous presence of nitrite and nitrate in the effluent, i.e. no full partial nitrification to nitrite was achieved in many of the studies reported (Fux et al., 2004). Nonetheless, there are also references in which stable continuous partial nitrification to nitrite have been obtained with artificial biofilms (Noto et al., 1998) and natural biofilms (Volcke et al., 2008). Additionally, Cecen and Gonenc, (1995) found that the combination of the bulk oxygen to bulk ammonium ratio is the most crucial parameter in the accumulation of nitrite. In nitrification, these researchers found a considerable degree of nitrite accumulation at bulk O_2 to bulk NH_4^+ concentration ratios lower than 5 mg O_2 (mg N)$^{-1}$. Bernet et al. (2005) found that using O_2/NH_4^+ ratio set points at 0.05 and 0.1 mg O_2 (mg N)$^{-1}$ it was possible to oxidize up to 80% of the inflow NH_4^+ into NO_2^-. Recently, Bartrolí et al. (2010) successfully obtained stable full nitritation by applying a DO/NH_4^+ ratio control strategy at set points lower than 0.25 mg O_2 (mg N)$^{-1}$.

iii) pH control: Based on the results reported by Anthonisen et al. (1976), NOB are more sensitive than AOB to FA and FNA. As a consequence, if pH in the reactor is increased (higher FA) or lowered (higher FNA), NOB inhibition will occur. Anthonisen et al. (1976) reported that FA initiated inhibition of *Nitrobacter* at about 0.1 and 1.0 mg N-NH_3 L^{-1}, while the threshold value for *Nitrosomonas* was 10 and 150 mg N-NH_3 L^{-1}. They also reported that FNA at a concentration as low as 0.22 mg N-HNO_2 L^{-1} inhibited the activities of *Nitrobacter*. These inhibitions

are only significant when dealing with high-strength ammonium-concentration wastewaters because the inhibitory concentrations of FA and FNA are more pronounced than the occurring in urban wastewaters. Nevertheless, the adaptation of bacteria to inhibition after the long-term operation is the main drawback of a strategy based exclusively on this factor (Turk and Mavinic, 1989b)

iv) Inorganic carbon concentration: Nitrifying bacteria are autotrophic and obtained their carbon source from inorganic compounds and the deficit of this substrate should result in a decrease of the process rate. Inorganic carbon is also linked to the alkalinity requirements of the system due to acidification of the media related to the nitritation process. Therefore, bicarbonate is used for both, buffering and growing purposes. Gujer and Boller (1986) reported that an alkalinity of at least 75 mg $CaCO_3$ L^{-1} was needed to maintain nitrification rate of nitrifying biofilters treating municipal wastewaters. Moreover, the limitation of alkalinity is also included in the Monod kinetics of the activated sludge model no 2 (ASM2) (Henze et al., 2000). Some studies have proven that bicarbonate is a suitable parameter for controlling partial nitrification using the stoichiometrical bicarbonate/ammonium ratio (Galí et al., 2007; Ganigué et al., 2008). Nevertheless, Guisasola et al. (2007) and Wett and Rauch, (2003) reported that bicarbonate limitation could also reduce AOB activity. Even more, Torà et al. (2010) reported that under total inorganic carbon (TIC) limitation, both inhibitions of FA and FNA over AOB were higher, thus the stability of partial nitrification systems could be disrupted.

Anaerobic ammonium oxidation (Anammox)

Another possible path to remove ammonium from wastewaters with low COD/N ratios consists in the autotrophic nitrogen removal combining AOB and Anammox bacteria. For a long time, it was thought that ammonium oxidation only could take place aerobically, until Broda (1977) predicted the existence of chemolitoautotrophic bacteria capable of oxidize ammonium using nitrite as electron acceptor. Later, this was confirmed experimentally by Mulder et al. (1995). Anammox bacteria convert ammonium together with nitrite directly to nitrogen gas in the absence of any organic carbon source, following the reaction described in Eq. 1.13 (Strous et al., 1998). In this process, a small amount of nitrate is also produced in the anabolism of Anammox bacteria.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2H_2O \quad \text{(Eq. 1.13)}$$

Phylogenetic analysis showed that the Anammox bacteria is inside the order *Plantomycetales*, a division within the domain Bacteria (Mulder et al., 1995; Strous et al., 1999). The optimal temperature and pH of Anammox operation is 35 °C and 8, respectively. Anammox bacteria are characterised by low biomass production at 0.038 g VSS g^{-1} N and a slow growth rate with large doubling times, as long as, 11 days (Strous et al., 2002). The advantage of this low biomass productivity is the reduction of operation costs related to sludge handling in the WWTP. However, the slow growth rate of Anammox bacteria makes the start-up of these processes long and difficult (Vázquez-Padín, 2009). Other important characteristic of Anammox bacteria is the fact that they are inhibited by both oxygen and nitrite. Careful measures need to be taken to avoid the inhibition and consequently, to avoid the high risk of failure of the reactor.

CANON, OLAND and aerobic deamonification

The partial nitrification and the Anammox processes can also be carried out together in a single, aerated reactor. This combined process has been called by different names CANON (Third et al., 2001), OLAND (Kuai and Verstraete, 1998) and aerobic deamonificaiton (Hippen et al., 1997). CANON process has been tested extensively at laboratory scale (Sliekers et al., 2002, 2003). Although Anammox requires strict anaerobic conditions, nitrifiers and Anammox organisms are able to coexist under oxygen-limited conditions. Therefore, CANON would need process control to prevent nitrite build-up by oxygen excess under ammonium limitation i.e. in the case of fluctuation ammonium load (Jubany, 2007). The OLAND is described as a new process for one step ammonium removal without the addition of COD (Kuai and Verstraete, 1998). Recently, it was confirmed that OLAND is based on the CANON concept (Philips et al., 2002). Aerobic deamonification is the direct conversion of ammonium into elemental nitrogen by autotrophic microorganisms. This method requires considerably smaller amount of carbon and oxygen (Hippen et al., 1997; Seyfried et al., 2001). Currently about 30 partial nitritation-Anammox applications are operating at full scale (Vlaeminck et al., 2012). In four of these, partial nitritation and Anammox are spatially separated (van der Star et al., 2007; Kampschreur et al., 2008; Desloover et al., 2011; Tokutomi et al., 2011), while in the others, a one-stage process is executed for reject water treatment (Joss et al., 2009; Weissenbacher et al., 2010), for landfill leachate treatment (Hippen et al., 2001; Denecke et al., 2007) and for industrial wastewaters (Abma et al., 2010).

With the development of biofilms, aerobic and anoxic zones can co-exist within the biofilm due to the oxygen gradients generated by the oxygen consumption of aerobic microorganisms. This will allow the development of AOB in the aerobic layers and Anammox bacteria in the anaerobic ones. In those systems, NOB growth has to be avoided since the oxidation of nitrite to nitrate is not desired. NOB compete for oxygen with the aerobic AOB and for nitrite with Anammox bacteria, and thus its growth should be prevented, for instance by controlling the DO concentration in the bulk liquid (Rosenwinkel and Cornelius, 2005).

1.2.3 Mathematical modeling of biological nitrogen removal

Today, the activated sludge processes are much more complex following their applications in single-sludge systems expanding from COD removal to nitrification, denitrification and phosphorus removal (the phosphorus removal is not been discussed in this Ph.D. thesis) (Tchobanoglous et al., 2004). The reactions involved in these processes are carried out by different types of bacteria that include a mixture of heterotrophic bacteria, bacteria that use or not nitrate as electron acceptor, as well as, autotrophic nitrifying bacteria. Competition exists between various types of heterotrophic bacteria for carbonaceous substrates. Furthermore, the importance of the wastewater components with regard to non-soluble, soluble, biodegradable and non-biodegradable substances on reaction rates, oxygen consumption and sludge production is better understood nowadays. Computer modeling provides an aid to evaluate activated sludge performance under both, dynamic and steady state conditions, and to design easily multiple-staged reactors, as well as, single complete-mix reactors by incorporating a large number of components and reactions representing the biological processes. Several simulation programs can be utilized including AQUASIM, WEST, Plan-It STOAT, BioWin, DESASS and the self development of models in MATLAB® environment.

The models may be used for a number of purposes (Tchobanoglous et al., 2004): i) as a research tool to evaluate biological processes and to better understand important parameters that affect certain type of performance; ii) as design tool for wastewater treatment plants; iii) as a evaluation and forecast tool for calculate the treatment capacity of a given facility and iv) as a tool for the development of new process control strategies by investigating the system response to a wide range of inputs without endangering the actual system. To obtain precise and reliable outputs in modeling, the accurate and representative wastewater characterization and calibration parameters are critical.

A long list of complex equations would be needed to describe the various reactions in an activated sludge process involving numerous components such as organic substrates (soluble and particulate), inorganic substrates (ammonium, nitrite, and nitrate), dissolved oxygen and various heterotrophic and autotrophic bacteria. The convenient way to represent the biokinetic models is using a table format, named in practice Gujer matrix or Petersen matrix (Takács, 2005). This table contains the stoichiometric matrices, kinetic vectors and state variables involved in the processes.

For the conventional floc-based activated sludge systems, the activated sludge model (ASM) established by the International Water Association (IWA), provides a consistent framework for the description of biological processes. The IWA developed four ASMs, the ASM1, ASM2, AMS2d and ASM3 (Henze et al., 2000). The ASM1 allows the simulation of organic matter removal and nitrogen removal (biological nitrification and denitrification) in activated sludge systems. The ASM2 is an extension of the ASM1 and includes biological phosphorous removal processes, and the ASM2d includes denitrifying phosphorous accumulating organisms (PAOs). Finally, the ASM3 is a new modeling platform based on recent knowledge of the activated sludge processes and includes the possibility of following up the concentrations of internal storage compounds. The decay process from ASM1 is replaced by an endogenous respiration process in ASM3, a more realistic approach under microbiological point of view (van Loosdrecht and Jetten, 1998).

In the case of biofilm or granular sludge systems, the guideline on the model selection developed by the IWA Task Group on Biofilm Modeling can be utilized as a basis for modeling and simulation of biofilm systems (Eberl et al., 2006). The model used by the Task Group can be grouped into four distinct categories according to the level of simplifying assumptions used; namely, analytical, pseudo-analytical, 1-D numerical and 2-D/3-D numerical. As a base line, normally all model types can represent biofilms with the following features: i) the biofilm compartment is homogeneous, with fixed thickness and attached to an impermeable flat surface, ii) only one substrate limits the growth kinetics, iii) only one microbial species is active, iv) the bulk liquid compartment is completely mixed and v) the external resistance to mass transfer of dissolved components is represented with a boundary layer compartment with a fixed thickness (Eberl et al., 2006). Moreover, biological processes in aerobic granules are determined by concentration gradients of oxygen and diverse substrates (Ni and Yu, 2010a). The substrate and DO concentration profiles are the result of many factors, e.g., diffusion coefficient, conversions rate, granule size, biomass spatial distribution and density. All of these factors tightly influence each other; thus the effect of separate factors cannot be studied experimentally. Model simulation and prediction, combined with model analysis can cope with this problem, together with techniques like uncertainty and sensitivity analysis, (Sin et al., 2009) providing a solid foundation for design and operation of biological treatment systems, including the aerobic granular sludge system, within the wastewater community.

The modeling of biofilm or granular structures is possible using one dimensional models (1-D) or multidimensional models (2-D and 3-D). The choice of the model is dependent on the model adequacy to the modeling objectives and to the environmental conditions of the WWTP to be modeled (Hauduc et al., 2012). The one dimensional model is simpler and therefore, requires less computational effort. Nevertheless, in 1-D models, several assumptions are made and it is assumed that the substrates gradients are some orders of magnitude higher in the perpendicular direction to the attachment surface than in the parallel plane to it (Vázquez-Padín, 2009). Important characteristics derived from the dynamics of biofilm structure e.g. external and internal mass transfer coefficients, changes in pore volume and motility of bacterial species inside the biofilm matrix (Xavier et al., 2005a) must be taken into account once assumed 1-D transport. On the other hand, 2-D and 3-D models are more complex and offer more potential to predict local compositions of particulate and dissolved variables.

Extensive research is being performed to model granular systems. For instance, Beun et al., (2001) described the COD and N removal in a granular sludge batch reactor using a 1-D model implemented in AQUASIM. These authors illustrated that nitrification, denitrification and COD removal can occur simultaneously in a granular sludge sequencing batch reactor SBR. Su and Yu, (2006) established a generalized model for aerobic granular SBR taking into account the removal of nitrogen and organic compounds, reactor hydrodynamics, oxygen transfer and substrates diffusion. Ni et al. (2008) used a modification of the ASM3 model to describe the simultaneous autotrophic and heterotrophic growth in aerobic granular SBR, utilizing the model in AQUASIM environment. de Kreuk et al. (2007) introduced the removal of phosphate, in addition to the removal of COD and N as a process in the 1-D model to study the influence of different parameters (oxygen concentration, temperature, granule size, sludge loading rate and cycle configuration) on a granular SBR operation. Vázquez-Padín et al. (2010) successfully described the biological processes of aerobic granular SBR based on the ASM platform with several modifications to model the granular reactor at variable COD/N ratios, including an accurate description of total solids concentration inside the reactor and biomass density. These authors also utilised a 1-D model implemented in AQUASIM software. The microbial product dynamics in aerobic granules in terms of formation of extracellular polymeric substances (EPS), soluble microbial products (SMP) and internal storage product were established, modelled and simulated by Ni and Yu, (2010b). This modeling study has elucidated the production and utilization of EPS, SMP and internal storage product and provide further insights into a granule-based SBR. Olivieri et al. (2011) developed a mathematical model of an aerobic biofilm reactor with double limiting substrate kinetics, following phenol inhibition and oxygen limitation on the suspended and immobilized biomass growth. The developed model was further used to investigate the bifurcational patterns and the dynamical behaviour of the reactor as a function of different key operating parameters. Mathematical models have been developed to analyze the complex 2-D and 3-D morphological and geometrical features of biofilms by van Loodstrecht et al. (2002), Picioreanu et al. (2004), Chambless and Steward, (2007), Xavier et al. (2005b; 2007) and Lewandowski et al. (2007)

1.2.4 Biological phenolic compounds removal

Phenolic compounds are among the substances present in large quantities in the wastewaters from petroleum, refinery, coke and chemical sectors. According to published data of the European Pollutant Release and Transfer Register (E-PRTR), the total amount of phenols released by the EU reporting states to E-PRTR was 1032 tonnes/year (phenols as total C) in

2010 (European Environment Agency (EEA), 2010). From this total amount of phenols released, about 74 % was contributed from petroleum, refinery, coke and chemical sectors.

The phenolic compounds present in the wastewater can be degraded by bacteria in aerobic or anaerobic processes. Since 1970s, information and knowledge related to the biodegradation of phenolic compounds has increased significantly, based on work with specific industrial wastewaters (i.e. petrochemical, chemical, pharmaceutical). In addition, since 1980s, work in these fields has expended knowledge on the capabilities and limitations of biodegradation. With a few exceptions, most organic compounds can be eventually biodegraded, but in some cases the rates may be slow, unique environment conditions may be required (i.e. redox potential, pH, temperature, acclimation time), or specific bacteria capable of degrading these compounds may be needed (Tchobanoglous et al., 2004).

In general, there are three principal types of degradation pathways that have been observed: i) the compound serves as a growth substrate; ii) the organic compound provides an electron acceptor; iii) the organic compound is degraded by co-metabolic degradation. In co-metabolic degradation, the compound that is degraded is not part of the microorganism's metabolism. Degradation of the compound is brought about by a nonspecific enzyme and provides no benefit for the cells to growth. Complete biodegradation of phenolic compounds to harmless products, such as CO_2 and H_2O or methane is always expected, however, in some cases, biotransformation to a different organic compound (may be innocuous, or just as harmful as or more harmful than the initial compound) is possible.

Phenolic compounds consist of a basic chemical structure containing phenol. During the biodegradation process, these compounds are following metabolic pathways similar to that of phenol. Aerobically, the biodegradation process requires the presence of molecular oxygen to initiate enzymatic attack on the aromatic rings. A typical pathway for metabolizing phenol is to hydroxylate the ring, by the enzyme phenol hydroxylase, form catechol, and then open the ring through *ortho*- (also termed β-ketoadipate pathway) or *meta*-oxidation (Jiang et al., 2006). Phenol hydroxylase represents the first enzyme in the metabolic pathway of phenol degradation.

Both *o*- and *m*-pathways are distinguishable by measuring their characteristic enzyme activities. In the *o*-pathway, the aromatic ring is cleaved by the enzyme catechol 1,2-dioxygenase (C120). In the *m*-pathway, the ring is cleaved by the enzyme catechol 2,3-dioxygenase (C230). Thus, the ring is opened and then degraded (Nair et al., 2008). The ring cleavage can occur in two different orientations, and this difference in cleavage site is used to classify catechol dioxygenases in two groups: the intradiol (such as C120) and extradiol (such as C230) cleaving enzymes (Cai et al., 2007). In the case of nitrophenol, the degradation of this compound could also oxidise to form hydroquinone and release nitrite, and later further complete degradation following the β-ketoadipate pathway (Ye et al., 2004). The main degradation route of *o*-cresol is following the 3-methyl catechol pathway (Masunaga et al., 1986). 3-methyl catechol was further degraded through at least two meta cleavage pathways. At this stage, a common route for catechol oxidation in the metabolic pathway of phenol is also applied in the *o*-cresol degradation pathway (Ribbons, 1966; Buswell, 1975). Discussion of biodegradation pathways and mechanism can be found in the literature (Masunaga et al., 1986; Ye et al., 2004; Kulkarni and Chaudhari, 2007; Nair et al., 2008).

Essential to anaerobic phenolic compounds metabolism is the replacement of all the oxygen-dependent steps by an alternative set of reactions and the formation of different central

intermediates (e.g. benzoyl-CoA) for breaking and cleaving the ring. Notable, in anaerobic pathways, the aromatic ring is reduced rather than oxidised. The two electron reduction of benzol-CoA to a cyclic diene is catalysed by benzoyl-CoA reductase. After nitrogenise, this is the second enzyme known which overcomes the high activation energy required for reduction of a chemically stable bond by coupling electron transfer to the hydrolysis of ATP. The alicyclic product cyclohex-1,5-diene-1-carboxyl-CoA is oxidised to acetyl-CoA via a modified β-oxidation pathway; the ring structure is opened hydrolytically (Heider and Fuchs, 1997). In some cases, phenolic compounds are anaerobically transformed to resorcinol or phloroglucinol.

Although aerobic and anaerobic microorganisms are able to degrade phenolic compounds, aerobic processes are preferred. Aerobic microorganisms are more efficient for degrading toxic compounds because they grow faster and usually transform organic compounds to inorganic compounds (CO_2, H_2O). Aerobic processes are also preferred due to the low costs associated with this option. This PhD thesis will also focus on the biological aerobic treatment of phenolic compounds.

Table 1.4 summarizes the results obtained by several recent studies related to the biological treatment of phenolic compounds by using either aerobic or anaerobic processes. The table also highlights the type of reactors used for each process.

Table 1.4 Recent studies on aerobic and anaerobic processes for the treatment of wastewater containing phenolic compounds. The full notation is presented at the end of the table.

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
Aerobic							
Phenol	synthetic	SPBR (1L)	activated sludge from municipal WWTP	-	Phenol, 83-100	OLR, 6	Lin et al. (2009)
Phenol	synthetic	MBSBR (8L)	activated sludge from municipal WWTP	-	Phenol, 99%	OLR, 4.5	Moussavi et al. (2009)
Phenol, COD NH_4^+	real (paper mill wastewater)	SBR (9L)	activated sludge from domestic WWTP	-	COD, 66-93 Phenol, 11-46 Nitrogen, 78-96	OLR, 1.33 NLR, 0.05	El-Fadel et al. (2012)
Phenolic mixture (phenol, pyridine, quinoline) NH_4^+	synthetic	RBC (4L)	activated sludge from dairy industry and mixed with specific degraders bacteria	-	COD, 91 Phenolic, 98 NH_4^+, 43	OLR, 1.3 NLR, 0.28	Jeswani and Mukherji (2012)
Phenol, cyanide NH_4^+	synthetic	1.CSTR (12L) 2.SBR (5L)	sludge from municipal WWTP	-	1.Phenol, 95 COD, 63-92 NH_4^+, 83-95 2.Phenol, 100 COD, 93 NH_4^+, 93-99	1.OLR, 3.2 NLR, 0.13 2.OLR, 2.6 NLR, 0.13	Papadimitriou et al. (2009)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
4-chlorophenol (4CP)	synthetic	1.SBR (2L) 2.MBR (3L)	activated sludge from municipal WWTP	sodium acetate	COD, 99 4CP, 100	1.OLR, 0.32 2.OLR, 0.16	Carucci et al. (2010)
4-chlorophenol (4CP)	synthetic	MBSBR (12.8L)	activated sludge from municipal WWTP	-	4CP, 65-100	OLR, 0.5-0.7	Lim et al. (2013)
4-chlorophenol (4CP)	synthetic	SBR (2.1L)	activated sludge from a cosmetic WWTP + bioaugmented with acclimated *Pseudomonas putida*	1.no co-substrate 2. phenol	4CP, 100	1.OLR, 0.03-0.5 2.OLR, 0.5	Monsalvo et al., (2012)
4-chlorophenol (4CP)	synthetic	SBR (2.1L)	industrial activated sludge WWTP	phenol	4CP, 100	OLR, 0.34	Monsalvo et al., (2009)
2,4-dichlorophenol (DCP)	synthetic	PCBR (2.3L)	activated sludge from WWTP of yeast production	sucrose	COD, 90 DCP, 90	OLR, 0.15	Dilaver and Kargi (2009)
2,4,6-trinitrophenol (TNP)	synthetic	BAF (17.6L)	isolated bacteria	-	COD, 95 TNP, 100	OLR, 2.3	Shen et al. (2009)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
p-nitrophenol (PNP)	synthetic	SBR (20L)	activated sludge from municipal WWTP	glucose	PNP, 100	OLR, 0.3	Martín-Hernández et al. (2009)
p-nitrophenol (PNP)	synthetic	PBR (4L)	isolated pure culture	-	PNP, 100	OLR, 1.87	Sahoo et al. (2011)
p-nitrophenol (PNP) 2,4-dinitrophenol (DNP) 2,4,6-trinitrophenol (TNP)	synthetic	SBRs (5L)	activated sludge from dairy WWTP and a pure culture	-	1.PNP, 98 2.DNP, 83 3.TNP, 84	1.OLR, 0.09 2.OLR, 0.07 3.OLR, 0.06	Kulkarni (2013)
Phenol and *m*-cresol	synthetic	ILALR (2.5L)	isolated mixed culture from sewage treatment plant	-	Phenol and *m*-cresol, 70-100	OLR, 4.2-8.4	Saravanan et al. (2009)
p-cresol NH_4^+	synthetic	SBR (5L)	lab scale nitrifying activated sludge	-	*p*-cresol, 100 NH_4^+, 99.7	NLR, 0.2 OLR, 0.75	Texier and Gomez (2007)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
2,4-dichlorophenol (DCP) 2,4,6-dichlorophenol (TCP)	synthetic	FBR (8.5L)	isolated bacteria from polluted rivers	-	COD, 89-92 DCP, 96 TCP, 97	OLR, 0.09	Gallego et al. (2011)
p-cresol NH_4^+ Sulfide	synthetic	CSTR (5L)	lab scale nitrifying activated sludge	-	*p*-cresol, 100 NH_4^+, 100 Sulfide, 100	NLR, 0.22 OLR, 0.09	Beristain-Cardoso et al. (2011)
p-cresol phenol *p*-hydroxybenzoate (P-OH) NH_4^+	synthetic	CSTR (5L)	lab scale nitrifying activated sludge	-	*p*-cresol, 99 phenol, 95 P-OH, 99 NH_4^+, 77-98	NLR, 0.22 OLR, 0.18	Pérez-González et al. (2012)
Anaerobic							
Phenol	synthetic	UAPB (2.8L)	activated sludge from pulp & paper industry	-	COD, 88 Phenol, 94	OLR, 2.5	Bakhshi et al. (2011)
Phenol	synthetic	AFBR (1.8L)	sludge from pig slurry	acetic, propionic, butyric	COD, 70 Phenol, 95	OLR, 5.03	Carbajo et al. (2010)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
Phenol	synthetic	AFBR (2L)	supernatant from fluidised bed reactor treating phenol	glucose	COD, 94 Phenol, 90	OLR, 5.3	Bajaj et al. (2009)
Phenol	synthetic	UASB (2.8L)	mesophilic phenol-degrading sludge from UASB reactor	-	Phenol, 99	OLR, 0.9	Fang et al. (2006)
Phenol, COD	real (coal gasification wastewater)	UASB (5L)	anaerobic sludge from coal gasification plant	-	COD, 55-60 Phenol, 58-63	OLR, 1.24	Wang et al. (2011a)
2,4-dichlorophenol (DCP)	synthetic	1.UASB (5.4L) 2.EGSB (5.4L)	anaerobic granular from lab scale UASB treating domestic wastewater and full scale UASB treating pulp bleaching wastewater	glucose	1.DCP, 75 COD, 61 2.DCP, 84 COD, 80	OLR, 0.13	Puyol et al. (2009)
p-nitrophenol (PNP)	synthetic	AMBR (13.5L)	anaerobic sludge from WWTP of yeast production	glucose	COD, 90-92 PNP, 92-94	OLR, 0.03	Kuşçu and Sponza (2009)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
Catechol	synthetic	UASB (9.75L)	digested sludge from domestic WWTP	glucose	COD, 95 Catechol, 82	OLR, 5	Subramanyam and Mishra, (2007)
4-chloro-2-nitrophenol (4C-2NP) 2-chloro-4-nitrophenol (2C-4NP) 2-chloro-5-methylphenol (2C-5MP)	synthetic	HUASB (7L)	anaerobic digester of slaughter house	glucose	COD, 65-98 4C-2NP, 90-97 2C-4NP, 83-93 2C-5MP, 74-90	OLR, 1-5	Sreekanth et al. (2009)
Phenolic mixture (phenol, *o,m,p*-cresol, dimethyl phenol)	synthetic	1.UASB (13.5L) 2.AHR (13.5L)	digester sludge from dairy industry and granular sludge from UASB treating distillery wastewater	-	1.COD, 85 Phenolic, 91 2.COD, 88 Phenolic, 93	OLR, 1.5	Ramakrishnan and Surampalli (2012)
Phenolic, fatty acid	real (olive mill wastewater)	IASB (2.5L)	anaerobic sludge from municipal WWTP	-	Phenolic, 60-81 COD, 76-89	OLR, 4,8	Gonçalves et al. (2012)

Table 1.4 *(continued)*

Target Compounds	Wastewater type	Reactor & volume	Inoculums used	Co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
Phenolic mixture	real (coal gasification wastewater)	UASB (1L)	anaerobic sludge treating coal gasification wastewater	-	COD, 50-55 Phenolic, 50-60	OLR, 1.1	Wang et al. (2011b)
Combined Aerobic-Anaerobic							
p-nitrophenol (PNP)	synthetic	AMBR/CSTR (13.5L)/(9L)	anaerobic sludge of UASB from yeast production (AMBR) activated sludge from yeast production WWTP (CSTR)	-	PNP, 91	OLR, 0.07	Kuscu and Sponza (2007)
Phenol, COD, NH_4^+	real (coking wastewater)	AO^2 biofilm (47L)	sludge from aeration tank of coking WWTP	-	COD, 90 Phenol, 100 NH_4^+, 99	OLR, 1.32 NLR, 0.3	Li et al. (2010)
Phenol, COD, NH_4^+	real (coal gasification wastewater)	A^2O-MBR (30L)	activated and anaerobic sludge from sewage treatment plant	-	Phenol, 99.7 COD, 97.4 NH_4^+ ,92.8	OLR, 1.8 NLR, 0.1	Wang et al. (2012)

NLR, nitrogen loading rate; OLR, organic loading rate (the value was calculated solely for phenolic loading rate); SPBR, spiral packed bed bioreactor; SBR, sequencing batch reactor; RBC, rotating biological contactor; CSTR, continuous stirrer tank reactor; MBR, membrane bioreactor; MBSBR, moving bed sequencing batch reactor; PCBR, packed column biofilm reactor; BAF, biological aerated filter; PBR, packed bed reactor; ILALR, internal loop airlift reactor ; FBR, fixed bed reactor; UAPB, upflow anaerobic packed bed; AFBR, anaerobic fluidized bed reactor; UASB, upflow anaerobic sludge blanket reactor; AMBR, anaerobic migrating blanket reactor; EGSB, expanded granule sludge bed; HUASB, hybric upflow anaerobic sludge; AHR, anaerobic hybrid reactor; IASB, inverted anaerobic sludge blanket; AO^2, anaerobic-aerobic-aerobic; A^2O, anaerobic-anoxic-oxic.

1.3 Aerobic granular sludge

Granular sludge technology, a novel environmental biotechnological process, is increasingly drawing attention of researchers engaging work in the area of biological wastewater treatment. Granular sludge is a self-immobilized microbial consortium, with a high density and includes biological, physical and chemical interaction phenomena (Liu and Tay, 2004). Granular sludge was first found in anaerobic upflow anaerobic sludge blanket (UASB) reactors to treat industrial wastewaters at the end of 1970s, and ten years after, it has been widely applied at full scales (Lettinga et al., 1980, Hickey et al., 1991). The anaerobic granular sludge technology exhibited several drawbacks that included a long start-up period, a relatively high operating temperature, unsuitability for low strength organic wastewater and low removal efficiency of nitrogen and phosphorous from wastewater (Adav et al., 2008). This resulted in the development of aerobic granular technology. Aerobic granular sludge is developed under aerobic conditions and mainly used for the aerobic degradation of organics and also for nitrogen removal under aerobic and anoxic conditions (Liu and Tay, 2004). In 2005, the IWA held the first seminar of aerobic granular sludge and a clear definition of aerobic granular sludge was established, i.e. granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs (de Kreuk et al., 2005a).

Aerobic granular sludge was first reported in an aerobic upflow sludge blanket reactor by Mishima and Nakamura (1991). Morgenroth et al. (1997) used a SBR to develop aerobic granular sludge and the granular sludge was formed after 40 days. Since that , SBR has been utilized quite often for aerobic granulation (Beun et al., 1999; Dangcong et al., 1999) and identified to be a suitable reactor configuration for this purpose (Liu and Tay, 2004). To date, most research work on the aerobic granulation has been conducted using SBR (Gao et al., 2011a). The research efforts have focused on the cultivation conditions, factors influencing granulation and the microbial community of the granular sludge. Laboratory research results have indicated that aerobic granular sludge has the following advantages compared to activated sludge;

i) Good settling properties. The settling velocity of aerobic granular sludge, depends on the size and density of the granules, reaching up to 50-90 m h^{-1}.
ii) A long sludge retention time of the flocculent sludge can be achieved, which is especially beneficial to nitrifiers and Anammox bacteria. Aerobic and anoxic zones are present inside the granules that can simultaneously perform different biological processes in the same system, for instance, simultaneous nitrification-denitrification (SND) could occur (Beun et al., 1999; Qin and Liu, 2006) and partial nitritation and the Anammox reaction were also observed in granular systems (Shi et al., 2009; Li et al., 2011).
iii) Aerobic granular sludge can be utilized to treat various wastewaters and is able to tolerate shock load due to the unique granular structure and high biomass concentration in the reactor (Adav et al., 2008, 2009; Maszenan et al., 2011). In addition, no sludge bulking was observed with the aerobic granular sludge (Moy et al., 2002).

The formation of aerobic granules consists of five stages; i) microbes' multiplication and microbe-to-microbe contact to form aggregates by hydrodynamic, diffusion, gravity and/or thermodynamic forces; ii) initial attraction to form aggregates and floc appearance by physical, chemical or biochemical forces; iii) floc cohesion through microbial forces to form

aggregates by biological glue like cellular clustering and secretion of extracellular polymeric substances (EPS); iv) hydrodynamic shear force to stabilize and to form mature granules and v) aerobic granule phase (Liu and Tay, 2002; Hailei et al., 2006). The formation of aerobic granules is a complex process influenced or controlled by several factors. The main factors are summarized as follow:

i) SBR operation: it appeared that aerobic granules were successfully cultivated only in SBR. The cyclic operation of SBR consisted of influent filing, aeration, settling and effluent discharge affects the aerobic granulation process and its final physical and chemical properties. Liu and Tay (2007) reported that the granules cultivated at 1.5 h cycle time were the biggest in size while the granules cultivated at 4 h cycle time were the most compact ones compared with those cultivated at other cycle times. A long starvation period weakened the granule stability (Wang et al., 2006a), whereas, intermittent and pulse feeding enhanced aerobic granulation and contributed to compact granules (McSwain et al., 2004). Furthermore, studies have indicated that short settling time could enhance aerobic granulation (Jiang et al., 2002; McSwain et al., 2004; Linlin et al., 2005). In terms of aeration intensity, Adav et al. (2007) reported that at low aeration intensity (1 L min^{-1}), no granules were formed. At high aeration rate, (3 L min^{-1}), mature and stable granules (1-1.5 mm) with a compact interior were formed. At intermediate aeration intensity (2 L min^{-1}), large granules (3-3.5 mm) with overgrown filaments were formed. Most aerobic granular sludge research was carried out at room temperature (20 – 25 °C). Besides cultivation of aerobic granules at low temperatures i.e. 8 °C seems to be not possible (de Kreuk et al., 2005b). Recently, Wang et al. (2012b) demonstrated that by applying an intensity of 48 mT static magnetic field, the time for aerobic nitrifying granulation could be enhanced from 41 to 25 days.

ii) Feed composition: various substrates were used to cultivate aerobic granules formulated in the synthetic influent wastewater such as glucose, acetate, phenol, starch, ethanol and others (Liu and Tay, 2002, 2004; Zheng et al., 2006; Adav et al., 2007). Aerobic granule cultivation with real wastewaters was also reported (Arrojo et al., 2004; Su and Yu, 2005; Wang et al., 2007a; Val del Río et al., 2012). Moreover, Jiang et al. (2003) revealed that the addition of Ca^{2+} ions accelerated aerobic granulation. Granules were formed in 16 days when 100 mg Ca^{2+} L^{-1} was added in the feed, whereas 32 days were required without the addition of Ca^{2+}. pH of the medium seems to be also a decisive factor during aerobic granulation. A slight alkaline pH around 7.5 is necessary for proper aerobic granulation, while granulation ceases to occur at pH above 8.5 (Hailei et al., 2006).

iii) Seed sludge: in most studies, aerobic granules were cultivated with activated sludge seed. The diverse bacterial community residing in activated sludge was important for aerobic granulation process since higher hydrophobic bacteria (likely to attach to sludge flocs) present in the seed sludge, the faster the aerobic granulation with excellent settleability (Wilén et al., 2008).

The characteristics of aerobic granules in term of physical (settling velocity, density, specific gravity, sludge volumetric index (SVI), chemical (specific oxygen utilization rate (SOUR), EPS) and biological parameters vary depending on several factors during the granule cultivation processes. In general, the settling ability of the sludge is an important parameter

which directly relates to the biomass retention capacity and the solid-liquid separation in the reactor (Gao et al., 2011a). The settling velocity varied from 50 to 90 m h^{-1}, even up to 130 m h^{-1} and was significantly higher than of sludge floc (7 to 10 m h^{-1}) (Qin et al., 2004; Zheng et al., 2006; Shi et al., 2009). The SVI, another parameter of sludge settling ability, of aerobic granules is generally below 80 mL g^{-1}, and even as low as 20 mL g^{-1} (Zheng et al., 2005; Gao et al., 2011b). Aerobic granules have a high density and compact structure, which helps in the biomass retention time, and solid-liquid separation. The density of aerobic granules varied from 12 and 125 g VSS $L_{particle}^{-1}$ depending on substrates introduced in the influent (Mosquera-Corral et al., 2003). The water content of the granules (about 94 and 97%), was lower than that (>99%) of flocculent sludge (Linlin et al., 2005). The aerobic granules had a very wide average diameter size range from 0.2 up to 16 mm (Beun et al., 1999; Zheng et al., 2005; Gao et al., 2011b). The size distribution of the aerobic granules was related to the operational conditions. Nevertheless, the DO was a major limiting factor for metabolic activity of the aerobic granules with a size larger than 0.5 mm. The diffusion limitations of the substrate could not occur for aerobic granules with a size less than 0.4 mm, and the substrate removal rate by granules with a size of 0.5 mm was almost three times than that of granules of a 1 mm size (Linlin et al., 2005; Li et al., 2008). Xiao et al. (2008) indicated porosities for bacterial granules ranging from 0.68 and 0.92, and the porosity rose as the size of granules increased.

One of the important chemical properties of aerobic granules is the content of extracellular polymeric substances (EPS). EPS is a gel-forming material secreted by cells, involved in the adhesion phenomenon, formation of a matrix structure, microbial physiology and improvement of long-term stability of granules (Wang et al., 2006b). EPS contains a variety of organic substances, such as polysaccharides, proteins, DNA, humic acid and uronic acid; affects the surface properties of cells, including surface charge and hydrophobicity; enhances polymeric interaction and promotes aerobic granulation (Wang et al., 2006). It also served as a carbon and energy source during starvation phase (since at least 50 % of the polysaccharide (PS) and 30 % of the protein (PN) in the EPS produced by aerobic granules are biodegradable), maintains the integrity of granules and forms a buffering layer against the harsh external environmental conditions. EPS is also known as a bioglue with a high polysaccharide content that could facilitate cell-to-cell interaction and further strengthen microbial structure through the formation of a polymeric matrix leading to aerobic granules (Liu et al., 2004). In contrast, Yu et al. (2009) reported that the EPS secreted by aggregated cells may retard cell-to-cell contact, thereby delaying granulation. This contradiction report between Liu et al. (2004) and Yu et al. (2009) is due to the fact that EPS may be hydrophilic or hydrophobic. When EPS contains uronic acids, such as D-glucoric acid, D-glactonic acid and D-mannuronic acid or hydrophobic proteins, it may be hydrophobic, enabling the microorganisms to attach to surfaces. Thus, EPS with a large content of uronic acid is hydrophobic and support aggregation (Khan et al., 2012).

The microbial structure of the granules is dependent on the inoculum, influent compositions, DO concentration and the size of the granules. In general, a granule could contain organic-degrading, nitrifying and denitrifying bacteria, as well as, anaerobic bacteria (Anammox, methanogens, etc.) if the granules were developed with sewage or a similar wastewater (Gao et al., 2011a). A conceptual aerobic granule is composed of three microbial communities, i.e. heterotrophic, aerobic bacteria could be grown on the outside, AOB in the middle and facultative and anaerobic bacteria in the core of the granules.

Aerobic granular sludge technology has been widely used for the treatment of various wastewaters, nutrient removal, organic toxic compounds degradation, dyestuffs, metal removal, etc. This technology is not only capable of treating single pollutant, but also multiple pollutants in wastewaters. A system like this would provide greater flexibility and control over the treatment process. In many cases, aerobic granular systems allow a more stable operation, the treatment of large organic loads, as well as, fluctuations in organic loads, remove multiple toxic pollutants, require small volumes for the settling systems and produce effluents of better quality than the conventional treatment systems (Khan et al., 2012). Recent researches related aerobic granular sludge application so far developed for the treatment of nitrogen, organic matter and phenolic compounds are summarized in Table 1.5. Additionally, since 2005 the first full-scale aerobic granulation technology (Nereda®) was already successfully applied for treatment of municipal wastewater (Zilverentant et al., 2011; van der Roest et al., 2012). Nowadays, over 10 full-scale systems of Nereda® technology owned by Royal Haskoning DHV were implemented for the treatment of both industrial and municipal wastewater (Royal HaskoningDHV, 2013). Another aerobic granular system, called ARGUS® was successfully developed and applied for treatment of industrial wastewater. ARGUS® is developed and owned by EcoEngineering Ltd, a company based in Croatia (Company EcoEngineering Ltd., 2013).

1.4 Future perspectives on simultaneous removal using aerobic granular sludge

At present although the research on aerobic granular sludge has made significant progress, future studies are still needed in order to fundamentally understand the granules behaviour and further development at a full scale system as a cost-effective technology. The fundamental of aerobic granulation process has been elucidated extensively (Adav et al., 2008; Gao et al., 2011a; Khan et al., 2012). The possibility of aerobic granules to perform simultaneous removal of nitrogen and organic matter co-existing within the same biomass aggregate is unquestionable. Biotreatment of toxic or recalcitrant compounds, including phenolic compounds by aerobic granular sludge also have been demonstrated. Although, several studies have demonstrated nitrifiers existing within the granule are sensitive and could be inhibited by the presence of phenolic compounds, few studies confirms the feasibility of simultaneous removal of both compounds. So that, the application of simultaneous removal of nitrogen and phenolic compounds using aerobic granules at laboratory, pilot or even at full scale is still scarce and needs more research attention.

In addition, the information about the stability of aerobic granular sludge in the presence of inhibitory compounds such as phenolic compounds or in front of shock load events or during long starvation periods either due to maintenance or production variations is still limited. This information is very valuable for the practical application of aerobic granular technology in treating various types of wastewaters, especially industrial wastewaters that are associated with process instability (substrates, load, toxicants and environmental parameters). The successfulness of simultaneous removal of nitrogen and phenolic compounds using aerobic granules will definitely offer several advantages for instance: installation and operation cost saving, process flexibility and system robustness. Hence, in the future, aerobic granular technology will be selected as one of the priority options for the implementation or expansion or upgrading of WWTPs either in municipal or industrial application.

Table 1.5 Recent researches on aerobic granular sludge application in treating wastewater containing nitrogen, organic and phenolic compounds. The full notation is presented at the end of the table.

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
Nitrogen and organic compounds							
NH_4^+	synthetic	SBR	OLAND biomass from lab-scale rotating contactor	-	Nitrogen, 50-80	NLR, 0.45	Vlaeminck et al. (2009a)
NH_4^+	real (black water)	RBC	OLAND biomass from lab-scale contactor	-	Nitrogen, 76	NLR, 0.7	Vlaeminck et al. (2009b)
NH_4^+	synthetic	SBR	activated sludge from an aeration tank of municipal WWTP	-	NH_4^+, 95	NLR, 0.5	Shi et al. (2010)
NH_4^+	synthetic	SBR	activated sludge from secondary sedimentation tank of municipal WWTP	-	NH_4^+, 90%	NLR, 0.05	Yao et al. (2013)
NH_4^+	synthetic	Air pulsing SBR	activated sludge from municipal WWTP	-	NH_4^+, 92	NLR, 0.25	Belmonte et al. (2009)

Table 1.5 *(continued)*

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
NH_4^+	synthetic	Airlift reactor	Activated sludge from urban WWTP	-	NH_4^+, 96-98	NLR, 0.75-6.1	Bartroli et al., (2010)
NH_4^+, COD	real (industrial wastewater from dairy, marine, fish canning, pig farm)	SBR	1.activated sludge from WWTP dairy products 2.activated sludge from plant processing marine products 3. activated sludge from urban WWTP	-	1.Nitrogen, 76; COD, 80 2. Nitrogen, 15; COD, 80 3. Nitrogen, 15 - 68; COD, 90 -93	1.NLR, 0.6; OLR 3.14 2. NLR, 0.22; OLR, 1.67 3. NLR, 0.15-0.62; OLR, 1.27-3.43	Val del Río et al. (2012)
NH_4^+, COD	real (diluted fish canning factory)	SBR	activated sludge from municipal WWTP	-	NH_4^+, 40; COD, 95	NLR, 0.18; OLR 1.72	Figueroa et al. (2008)
NH_4^+, COD	synthetic	GMBR	GAO granules cultivated from activated sludge in an anaerobic-aerobic SBR	sodium acetate	NH_4^+ ,42 -78; COD, 85 -92	NLR, 0.14-0.16; OLR, 1.8-4.2	Wang et al. (2008)
NH_4^+, COD	real (municipal)	SBR	activated sludge from an aeration tank of municipal WWTP	-	NH_4^+, 95; COD, 90	NLR, 0.19; OLR, 0.96	Ni et al. (2009)

Table 1.5 *(continued)*

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
NH_4^+, COD	synthetic	SBR	aerobic granular sludge from lab-scale SBR	sucrose	NH_4^+, 98; COD, 98	NLR, 0.32; OLR, 1.5	Shi et al. (2009)
NH_4^+, COD	real (40% domestic, 60% industrial)	SBR	activated sludge from an aeration tank of municipal WWTP	-	NH_4^+, 98; COD, 80	NLR, 0.43; OLR, 3.24	Liu et al. (2010)
NH_4^+, COD	synthetic	SBAR	1.activated sludge from beer WWTP 2.activated sludge from municipal WWTP	Glucose and peptone	1.NH_4^+, 94; COD, 94 2.NH_4^+, 93; COD, 95	NLR, 0.166; OLR, 3	Song et al. (2010)
NH_4^+, COD	real (swine slurry)	GSBR	activated sludge from municipal WWTP	-	NH_4^+, 70; COD, 87	NLR, 0.83; OLR, 4.4	Figueroa et al. (2011)
NH_4^+, COD	synthetic	SBR	partial nitrifying activated sludge	Glucose and sodium acetate	Nitrogen, 45-70; COD, 99	NLR, 0.9; OLR, 2.4	Wang et al. (2012a)
NH_4^+, COD	real (diluted pig slurry)	CSTR	1.activated sludge from municipal WWTP 2.slurry from pig farm	-	1.Nitrogen, 10-15; COD, 50-80 2.Nitrogen, 10-15; COD, 30-60	1. NLR, 0.9-2.2; OLR, 4.8-12 2. NLR, 1.4-1.7; OLR, 4.8-6	Morales et al. (2012)

Table 1.5 *(continued)*

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
NH_4^+, COD	real (diluted chemical industry wastewater)	SBR	activated sludge from an aeration tank of municipal WWTP	-	Nitrogen, 40; COD, 80	NLR, 0.2; OLR, 1	Liu et al. (2011)
Phenolic compounds							
Phenol	synthetic	GSBR	granular sludge from bioreactor treating phenol-laden wastewater	-	Phenol, 99	OLR, 1.7	Moussavi et al. (2010)
	synthetic	column-type SBR	activated sludge from municipal WWTP	-	Phenol, 99-100	OLR, 0.33	Adav et al. (2007a)
	synthetic	column-type SBR	activated sludge from municipal WWTP	-	Phenol, 100	OLR, 3.4	Jiang et al. (2010)
	synthetic	column-type SBR	aerobic activated sludge from municipal WWTP	-	Phenol, 80-95	OLR, 1.13	Adav et al. (2007b)
p-nitrophenol (PNP)	synthetic	SBR	acetate-fed pre-grown aerobic granular sludge	1.no co-substrate 2.acetate	PNP, 100	OLR, 0.25	Suja et al. (2012)
	synthetic	SBR	pre-cultivated acetate-fed aerobic granular sludge	1.no co-substrate 2.acetate 3.no co-substrate	PNP, 100	1.OLR, 0.05 2.OLR, 0.025 3.OLR, 0.22	Yarlagadda et al. (2012)

Table 1.5 *(continued)*

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
p-nitrophenol (PNP)	synthetic	column-type SBR	activated sludge from municipal WWTP	glucose	PNP, 99.9	OLR, 1.02	Yi et al. (2006)
o-nitrophenol (ONP)	synthetic	SBR	activated sludge from municipal WWTP	glucose	ONP, 78	OLR, 0.18	Basheer et al. (2012a)
m-cresol (MC)	synthetic	SBR	activated sludge from municipal WWTP	-	MC, 87	OLR, 3	Basheer and Farooqi (2012b)
p-cresol (PC)	synthetic	SBR	activated sludge from municipal WWTP	-	PC, 96	OLR, 3	Basheer and Farooqi (2012c)
Phenol and *m*-cresol	synthetic	SBR	aerobic digested sludge from paper mill	-	Phenol, *m*-cresol, 95	OLR, 7.1	Farooqi et al. (2008)
2-chlorophenol (2CP)	synthetic	SBR	Aerobic sludge from paper mill	glucose	2CP, 100	OLR, 0.7	Khan et al. (2011a)
2,4,6-trichlorophenol (TCP)	synthetic	column-type SBR	aerobic sludge from secondary clarifier of municipal WWTP	1.glucose 2.sodium acetate	1. COD, 98; TCP, 100 2.COD, 96 TCP, 100	1.OLR, 0.61 2.OLR, 0.51	Khan et al. (2011b)

Table 1.5 *(continued)*

Compounds	Wastewater type	Reactor	Inoculums used	Carbon source or co-substrate	Removal efficiency (%)	Loading rate NLR (g N L^{-1} d^{-1}) OLR (g COD L^{-1} d^{-1})	Reference
2,4,6-trichlorophenol (TCP)	synthetic	GSBR	4CP-fed aerobic granular sludge	sodium acetate	TCP, 100	OLR, 0.07	Carucci et al. (2008)
2,4-dichlorophenol (DCP)	synthetic	column-type SBR	activated sludge from secondary clarifier of municipal WWTP	glucose	DCP, 94	OLR, 2.8	Wang et al. (2007b)
2,4-dichlorophenol (DCP)	synthetic	SBR	aerobic sludge from secondary clarifier of paper mill	-	DCP, 95	OLR, 0.45	Khan et al. (2011c)
4-chlorophenol (4CP)	synthetic	GSBR	acetate-fed aerobic granular sludge	sodium acetate	4CP, 100	OLR, 0.32	Carucci et al. (2008, 2010)
2-fluorophenol (2FP)	synthetic	SBR	aerobic granular sludge treating sewage	sodium acetate	2FP, 100	OLR, 0.06	Duque et al. (2011)

NLR, nitrogen loading rate; OLR, organic loading rate (for phenolic compounds, the value was calculated solely for phenolic loading rate); SBR, sequencing batch reactor; RBC, rotating biological contactor; GMBR, granular membrane bioreactor; SBAR, sequencing batch airlift reactor; GSBR, granular sequencing batch reactor;

1.5 References

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Chapter 2

Objectives

Summary

In this chapter, the problem statement is defined following the state-of-the-art review presented in the previous chapter. This allows to define the main goal of the thesis, as well as, several working objectives derived from this main goal.

2.1 Problem statement

Several industrial sectors for instance petrochemical, refinery, coke processing and chemicals, are producing complex wastewaters characterized with a high concentration of ammonium and phenolic compounds. Such wastewaters are treated by means of physicochemical and biological processes. The physicochemical methods are associated with high capital and investment costs and for being energy intensive processes. Besides, these processes do not completely remove the contaminants. Frequently, a conventional biological treatment is incapable to treat these wastewaters due to the toxicity and inhibitory impact of phenolic compounds on the biological processes. Moreover, as response to changes in the production schedule, biological treatment could be exposed to sudden changes in the waste streams profile in terms of concentration, inflow and presence of toxic contaminants, this would create extra-difficulties in the biological treatment facility. In most cases, the biological processes are inhibited by the dynamic changes and the presence of phenolic compounds; thus, deteriorate the wastewater treatment plant (WWTP) efficiency and consequently affecting the quality of the discharged effluent. There is an urgent need either to modify or to innovate existing biological treatment technologies to enhance their capability to simultaneously eliminate nitrogen and phenolic compounds. Also, the proposed technologies should be able to maintain a high efficiency operation during dynamic or transient periods. The application of aerobic granular sludge technology could be an excellent alternative since it has a lot of advantages in terms of associated costs, space footprint requirement and removal efficiency, among others. The unique microbial structure of the granular sludge provides a conducive platform for nitrifiers (able to oxidise ammonium) and heterotrophs (able to remove phenolic compounds) to coexist within a single granule. The removal of ammonium via 'nitrite route' and subsequent process either by heterotrophic denitrification or Anammox offers various advantages such as significantly save in aeration and external carbon sources requirements and a reduced production of greenhouse gas emissions than the conventional process.

2.2 Objectives

The main objective of this PhD thesis was to perform a feasibility study of simultaneous partial ammonium oxidation to nitrite (nitritation) and phenolic compounds removal using aerobic granular sludge reactors operating in continuous mode. The working objectives started at a lab-scale level and focused on the acquisition of basic knowledge related to the partial nitritation of synthetic high-strength ammonium wastewater. The basic knowledge gained was applied in a modeling study and parameters affecting the partial nitritation process using continuous granular sludge reactors were simulated and investigated. The following step was to complete an experimental feasibility study of simultaneous nitritation and phenolic compounds removal using aerobic granular reactors. Finally, the stability and the performance of simultaneous removal processes under operational instabilities were assessed by conducting several scenarios related to inflow instability such as shock loads, sequentially alternating pollutants (SAP) and starvation periods.

In order to achieve the main goal, the following specific objectives were established:

- To model mathematically the process of nitritation in aerobic granular reactors operating in continuous mode aiming to:

- To implement the [DO]/[TAN] ratio control strategy for obtaining stable nitritation.
- To validate the developed model with experimental results obtained from nitrifying granular reactors at laboratory and pilot plant scale treating high-strength concentration ammonium wastewaters.
- To study the effect of DO and TAN setpoints, operating temperature, biofilm characteristics (granules size, density) and ammonium concentrations in the influent on the achievement of full nitritation by applying the [DO]/[TAN] ratio control.

❖ To explore the use of bioaugmentation as a tool to develop a granular sludge able to degrade recalcitrant compounds and simultaneously performing nitritation, aiming to:

- To study the feasibility of simultaneous partial nitritation and *o*-cresol removal using an aerobic granular reactor operating in continuous mode.
- To study the stability of the simultaneous partial nitritation and *o*-cresol removal in front of shock load events.
- To study the feasibility of simultaneous nitritation and *p*-nitrophenol (PNP) removal using a continuous aerobic granular reactor.
- To study the impact on the biomass characteristics of the abovementioned compounds in their simultaneous removal processes.

❖ Finally, to study the stability and performance of the simultaneous nitritation and phenolic compounds removal under transient state, aiming to:

- To study the performance of aerobic granular reactor performing simultaneous partial nitritation and *o*-cresol removal in front of SAP scenarios.
- To study the impact of long-term starvation periods (in one of the two substrates (PNP) and total starvation of both substrates (PNP and TAN)) and subsequent reactivation of the simultaneous nitritation and PNP removal using a continuous aerobic granular reactor.

Chapter 3

Materials and Methods

Summary

In this chapter, the experimental set-up, analytical and biomass characterization methods used in this work are described in detail. Besides, the microbial identification technique i.e. fluorescent in-situ hybridization (FISH) is also explained. However, the specific materials and methods used for instance, the software in modeling, the inoculums used, the feed compositions and the strategy in each of the experimental studies are addressed in detail in its corresponding chapter.

3.1 Experimental set-up

To study the nitritation process in aerobic granular reactors operating in continuous mode by means of mathematical modeling , the experimental findings in Bartroli et al. (2010) obtained from a pilot plant granular airlift reactor (112 L) treating a high-strength ammonium-concentration synthetic wastewater were exploited (Chapters 4 and 5). Additionally, the experimental results obtained from a 150 L pilot plant granular airlift reactor treating real reject water were also used in the modelling study presented in Chapter 5 (Torà et al., *under review*). Figure 3.1A and 3.1B depict the two aerobic granular sludge reactors at pilot scale utilized in the whole modeling study (Chapter 4 and 5). Both reactors were equipped with pH, dissolved oxygen (DO), temperature and NH_4^+ probes. Compressed air was supplied through an air diffuser placed at the bottom of the reactor. The pH was maintained in the reactor bulk liquid through the addition of solid Na_2CO_3. Two different closed-loops control strategy were implemented in each of the reactor i) A closed feedback control loop was implemented to maintain the total ammonium nitrogen (TAN) concentration at a desired setpoint and ii) a proportional-integral (PI) controller was implemented to control the DO concentration in the bulk liquid. Both control strategy were integrated and called ratio control ($[DO]_{sp}$ / $[TAN]_{sp}$). The details of the ratio control strategy implemented on the reactors were described in chapter 4 & 5.

In the experimental study presented in chapter 6, 7, 8 and 9, a laboratory scale airlift reactor (2.6 L) was utilised to investigate the simultaneous nitritation and phenolic compounds removal operating in continuous mode. The reactor was equipped with a data acquisition system (pH, DO, temperature), feeding pumps, a temperature controller, pH and DO probes. Compressed air was supplied through an air diffuser placed at the bottom of the reactor. pH inside the reactor was maintained by the addition of Na_2HCO_3. The experimental results obtained during the reactor start-up and achievement of partial nitritation process i.e. prior to the simultaneous nitritation and phenolic compounds removal experiments, were used in the modeling study of nitritation (Chapter 5). Figure 3.1C depicts the laboratory scale airlift reactor utilized in the study. Besides, Table 3.1 summarized the description and experimental set-up of each of the reactors used in the whole thesis.

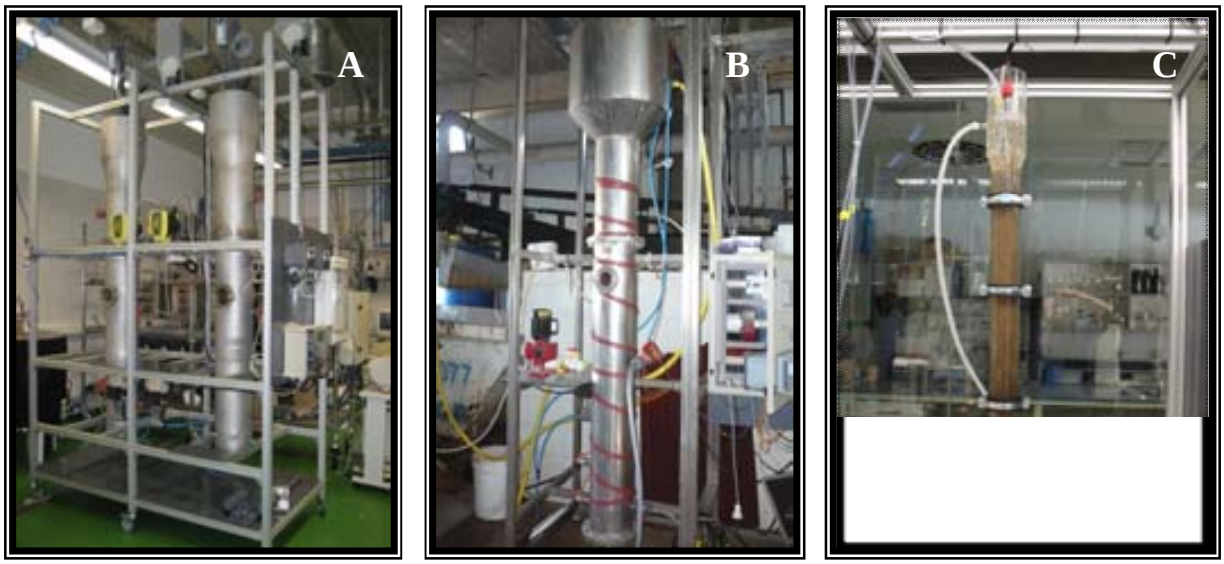

Figure 3.1 Aerobic granular sludge airlift reactors employed in the experimental study. A. Pilot plant reactor, 112 L, ; B. Pilot plant reactor, 150 L, C. Lab scale reactor, 2.6 L.

Table 3.1 Reactors volume, type and main characteristics of wastewater, inoculums used and biological processes performed in each of the airlift reactors utilised in the study. Total ammonia nitrogen (TAN); chemical oxygen demand (COD); total organic carbon (TOC); total inorganic carbon (TIC); *p*-nitrophenol (PNP); 2-chlorophenol (2CP).

Exp. set-up	Reactor volume (L)	Wastewater type	Main characteristics of wastewater (mg L^{-1})	Inoculum	Related Chapter
A	112	Synthetic. High-strength NH_4^+ concentration	TAN; 1200 COD; 30	Activated sludge	Chapters 4 and 5: Modeling of nitritation in granular reactors
B	150	Real reject water	TAN; 500 to 850 TOC; 240 to 696 TIC; 358 to 753	Activated sludge	
C1	2.6	Synthetic. High-strength NH_4^+ concentration	TAN; 950 COD; 30	Aerobic granular sludge	
C2	2.6	Synthetic. High-strength NH_4^+ concentration	TAN; 950 COD; 30 to 2500[a] *o*-cresol; 5 to 1000[b] PNP; 5 to 15[b] Phenol; 15[c] 2CP; 15[c]	Aerobic granular sludge[d]	Chapter 6: Simultaneous nitritation and *o*-cresol removal. Chapter 7: SAP scenarios Chapter 8: Simultaneous nitritation and PNP removal Chapter 9: Starvation

[a] the concentration of COD is depending on the concentration of the phenolic compound included in the wastewater
[b] the concentration of *o*-cresol and PNP was variable depending on the particular experiment.
[c] Concentration of each compound used in the study of sequentially alternating pollutant (SAP) scenarios .
[d] the granular sludge was treating low-strength wastewater to remove COD, nitrogen and phosphorus, later bioaugmentation was applied after achievement of nitritation, and prior the addition of phenolic compounds.

3.2 Wastewater characteristics

The main influent used in the experimental set-up A, C1 and C2, consisted of a synthetic wastewater with a high-strength TAN (TAN= $N\text{-}NH_4^+$ + $N\text{-}NH_3$) concentration and a low

concentration of biodegradable COD. The detail of the components utilised in the preparation of the synthetic wastewater in experimental set-up A, C1 and C2 is shown in Table 3.2. In addition, in experimental set-up B, the concentration of real reject wastewater fed into the 150 L granular airlift reactor was rather variable depending on the efficiency of the anaerobic digestion process (Table 3.1). In the study of simultaneous nitritation and phenolic compounds removal (experimental set-up C2), the main synthetic wastewater was added with phenolic compounds in variable concentrations depending on the particular experiments (see Table 3.1). Details on the influent compositions used in each of the experiment is described in each of the chapter.

Table 3.2 Composition of the main synthetic wastewater used in the experimental set-up A, C1 and C2 described in Table 3.1.

Component	Composition (mg L^{-1})
NH_4Cl	3630 to 4600 (950 to 1200 mg N-TAN L^{-1})
CH_3COONa	48
$CaCl_2 \cdot 2H_2O$	3
KH_2PO_4	13.0
NaCl	9.0
$MgCl_2 \cdot 7H_2O$	6.0
$FeSO_4 \cdot 7H_2O$	0.13
$MnSO_4 \cdot H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.13
$CuSO_4 \cdot 5H_2O$	0.07
H_3BO_3	0.007

3.3 Analytical methods

Regular sampling of the bulk liquid of the reactor (experimental set-up A, B, C1, C2) was carried out to determine chemical parameters off-line such as TAN, total nitrite nitrogen (TNN= $N\text{-}NO_2^-$ + $N\text{-}HNO_2$), nitrate and phenolic compounds in experimental set-up C2 (phenol, *p*-nitrophenol, *o*-cresol, 2-chlorophenol).

The ammonium concentration measured as total ammonia nitrogen (TAN) was analyzed using a continuous flow analyzer based on potentiometric determination (Baeza et al., 1999). The nitrite and nitrate were measured with ionic chromatography (Fig. 3.2A) using an ICS-2000 Integrated Reagent-Free IC System (DIONEX) with an IonPac AS9-HC column and an Anion Self-Regenerating Suppressor in autosuppression recycle mode (ASRS ULTRA II 4 mm).The samples were filtered using 0.22 μm filter units. Eluent solution consisted of KOH (DIONEX) at 10 mM from 0 to 10 min and 10-45 mM from 10 to 25 min. The conditions were 30 °C, 25 μL of volume injection, 1 mL min^{-1} of flow injection, and 25 min of analysis time.

o-Cresol, PNP, phenol and 2CP were determined by High Performance Liquid Chromatography (HPLC) (UltiMate 3000, Dionex Corporation) using an Agilent Zorbax SB-

C18 (4.6 x 100 mm, 3.5 μm) column and a UV detector set at 254 nm, the flow rate was 1.875 mL min^{-1} and the column temperature was maintained at 30 ºC (Fig. 3.2B). The mobile phases were ultrapure water containing H_2SO_4 at pH 1.41 and HPLC grade methanol following a gradient elution. The gradient started from 100% of acidified water and progressively changed to 50:50 v/v of water:methanol in 18 min, then it remained isocratic until 20 min. The injection volume was 20 μL and the maximum pressure in the column was approximately 290 bars (Martín-Hernández et al., 2009).

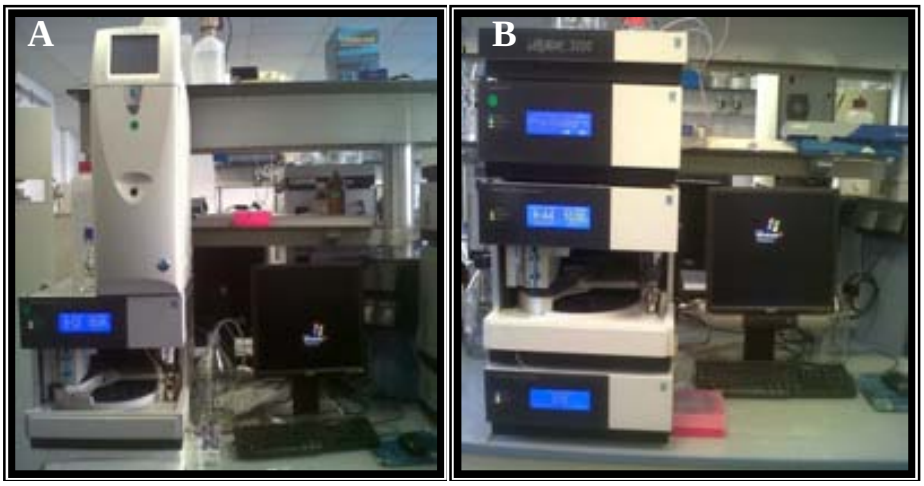

Figure 3.2 Analytical equipments utilized in the experiment. A. Ion chromatography. B. High Performance Liquid Chromatography (HPLC).

Total suspended solids (TSS) represent the total amount of inorganic and organic matter in the mixed liquor sample. Volatile suspended solids (VSS) correspond to the volatile organic matter which is close to the amount of biomass. Both parameters were analysed according to Standard Methods (APHA, 1998). In brief, 100 mL of well-mixed sample is added into a dish previously dried up and weighed (W_0). The dish was placed in an oven at 104 °C overnight and putted in the desiccator for 2 hours before weighing it again (W_1). Later, the dish was put in a furnace using a ceramic bowl for 30 minutes at 550°C and then, in the desiccator for 2 hours prior weighing it (W_2). The difference between W_0 and W_1 indicates the TSS. Conversely, VSS would be the result of the difference between W_1 and W_2. Both parameters were expressed as mass per volume of sample (i.e., g L^{-1}). Triplicate samples were done. Mixed liquor from the reactor and effluent were sampled regularly for TSS and VSS analysis. For an effluent sample, the procedure in the first step was slightly changed instead of using a dish, a glass fibre filter paper (Whatman) was employed, and it was placed in the oven for only 2 hours. The remaining TSS and VSS determination procedure was similar.

3.4 Biomass characterization

The granular biomass was characterized in term of size, granule density, settling velocity and extracellular polymeric substances (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 of granules to black and white for the image processing, (ii) to define the threshold 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 determination, minimum of 20 images were captured, and at least 20 to 40 granules were present in each image.

The density of the granules biomass was determined using the dextran blue method described by Beun et al. (2002). In brief, a known amount of a homogeneous granular biomass sample was taken from the reactor. Then, a known amount of liquid was removed from the sample. Then, a known volume of dextran blue solution (1 g L^{-1}) was added to a representative sample (and known amount) of granular sludge, in a volumetric ratio of about 1:1. The mixture was gently mixed, and subsequently the granules were allowed to settle down. A known amount of the liquid above the settled granules was removed, and a sample was taken from it. This fraction and the original dextran blue solution were analyzed by a spectrophotometer at 620 nm. Subsequently the volume occupied by the granular biomass in the reactor sample was calculated, since dextran blue only binds to water and not to biomass. By multiplying the volume of sample taken from the reactor with VSS and dividing by the calculated biomass volume, the density of the granules as g biomass per L of granules can be determined. 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). At least 50-70 granules were measured, and an average of settling velocity was calculated.

The sludge volumetric index (SVI) was measured according to Standard Methods (APHA, 1998). SVI is defined as the volume in millilitres occupied by 1 g of suspension after 30 min settling. However, de Kreuk et al. (2005) and Schwarzenbeck et al. (2004) proposed another parameter, the SVI_5 (SVI after 5 minutes of settling) to be used together with the SVI_{30} (SVI after 30 minutes of settling) since the current definition to discern between a granule and a floc is that granules settle significantly faster than flocs and do not coagulate under reduced shear stress (de Kreuk et al., 2005). Hence, it is considered that granular sludge should have a SVI_5/SVI_{30} ratio close to 1 which means that no significant compaction of sludge bed occurs after settling.

The EPS were extracted from the granules using formaldehyde + NaOH according to Adav and Lee (2011). In brief, 50 mL of sample from the reactor was taken, and the granules were left to settle. Supernatant was discarded, and the granules were washed with mili-Q water. The granules were crushed using a mortar and a pestle. 10 mL of the crushed granules sample was added with 0.06 % formaldehyde. The sample was kept in 4 °C for 1 hour. Then, 4 mL of NaOH (1M) was added into the sample and kept in 4 °C for 3 hours. Later, the supernatant from the sample was taken and centrifuged at 4 °C, 10 000 rpm and 30 minutes. The EPS extracted sample (supernatant) was recovered and further analysed for polysaccharides and protein contents. The polysaccharides content in the EPS extracted sample was determined using a colorimetric method with glucose as standard (Dubois et al., 1956). The protein content in the extracted sample was measured using the Lowry method with bovine serum albumin as standard (Lowry et al., 1951; Gerhardt et al., 1994).

3.5 Microbiological ecology assessment

The fluorescence in-situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM) was used to identify possible heterotrophic bacteria able to degrade phenolic compounds, betaproteobacteria ammonia-oxidizing bacteria (β-AOB) and nitrite oxidizing bacteria (NOB) in the granules. According to Amann et al. (1995) a typical FISH protocol includes four steps (Fig. 3.3); i) the fixation and permeabilization of the sample; ii)

hybridization of the targeted sequence to the probe; iii) washing steps to remove unbound probe; and v) the detection of labelled cells by microscopy. Leica TCS-SP5 AOBS confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was used. The microscope was equipped with a HC PL APO CS 63x1.25 oil objective, with two He-Ne lasers and a hybrid detector. Hybridization was carried out using the probes targeting specific microorganisms as described in Table 3.3. The general probe consisting of equal parts of UNIV1390 and EUBmix was used for detection of all bacteria in the granules. FISH protocol for heterotrophic bacteria able to degrade phenolic compounds was followed according the procedures highlighted by Suárez-Ojeda et al. (2011). The probes used for heterotrophic bacteria were selected taking into account the characterisation previously done by these authors of a PNP-degrading biomass used in this PhD thesis for developing granules with simultaneous capabilities for nitritation and phenols removal. The quantification of the microbial populations was performed following a modification of the procedure described in (Jubany et al., 2009). Prior to the quantification, the granular biomass was crushed using a mortar and a pestle and then typical FISH procedures highlighted by Suárez-Ojeda et al. (2011) was followed. To apply the quantification methodology to granular biomass (crushed), 40-50 microscopic fields were analyzed, and a single z-position was selected based on the highest intensity for each granule sample. Two images were generated from each field: one using the appropriate laser to detect the specific probe (SP image) and the other one, using the appropriate laser for the general probe (GP image). Prior to the quantification, the thresholds of the obtained image were corrected accordingly. The comparison between GP image and SP image is processed automatically in Matlab® environment and finally, the quantification result is taken directly from the output produced.

In order to assess the spatial position of the identified bacteria in the granules, some aggregates were cut in slices. In brief, several granules were put in the centre of cassette having biopsy pads on both side and leaved completely submerged in buffered formaldehyde 4 % for 24 h or more. The cassette in which consists of granules was 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 slices. Hybridizations were performed with the protocol abovementioned and the probes targeting specific microorganisms as described in Table 3.3 were used. In order to obtain a better granule staining, the amount of probe used in the hybridizations step was augmented 4 to 5 times depending on the total area of sliced granule. Figure 3.4 illustrates the protocol for assessment the spatial position of the identified bacteria in the granules.

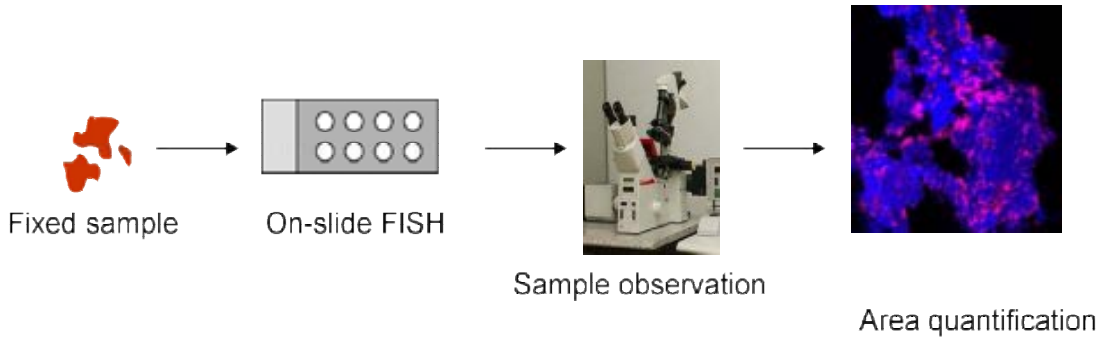

Figure 3.3 Typical steps in fluorescence in-situ hybridization (FISH) protocol.

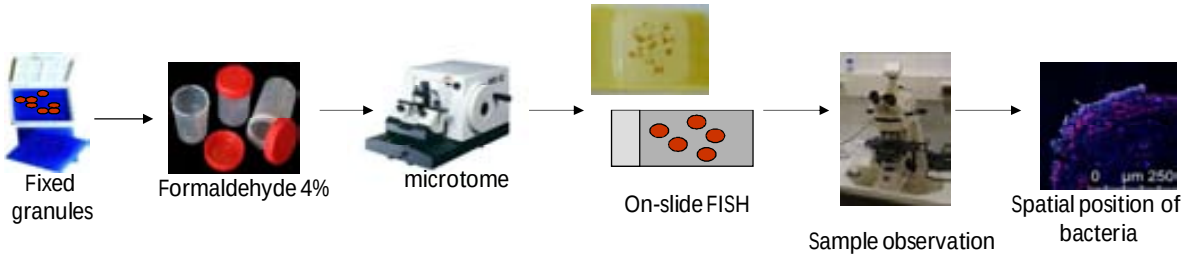

Figure 3.4 The protocol applied for assessment the spatial position of the identified bacteria in the granules.

Table 3.3 Probes targeting specific microorganisms employed in the FISH analysis

Probe name	Specificity	Reference
Nso190	β-ammonia oxidizing bacteria	Mobarry et al. (1996)
NIT3	*Nitrobacter* sp.	Wagner et al. (1996)
KO 02	*Arthrobacter* sp.	Franke-Whittle et al. (2005)
ACA652	Genus *Acinetobacter*	Wagner et al. (1994)
UNIV1390	All organisms	Zheng et al. (1996)
EUBmix*	Most bacteria, planctomycetales and verrucomicrobiales	Daims et al. (1999)

*EUBmix is a mixture of EUB388, EUB388 II, EUB388 III

3.6 References

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PART II

MODELING OF NITRITATION IN GRANULAR REACTORS

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Chapter 4

Closed-loop control of ammonium concentration in nitritation: convenient for reactor operation but also for modeling

Summary

A mathematical biofilm model was developed to describe nitritation in aerobic granular reactor operating in continuous mode. The model includes the automatic closed-loop control of ammonium concentration in the effluent. This is integrated in a ratio control strategy to maintain the proportion between the dissolved oxygen (DO) and the total ammonia nitrogen (TAN) concentrations in the reactor effluent at a desired value. The model was validated with a large set of experimental results previously reported in the literature. The model was used to study the effect of DO and TAN setpoints on the achievement of full nitritation, as well as to establish the appropriate required range of the DO/TAN concentration ratio to be applied. Nitritation at 20 ºC was tested experimentally and simulated with the model. Additionally, the importance of controlling the TAN concentration was highlighted with different scenarios, in which periodic disturbances were applied mimicking a poor control situation.

4.1 Introduction

Nitritation is a key process for the correct performance of the biological nitrogen removal via nitrite. When a separate reactor is devoted to nitritation, ammonium concentration in the reactor is of fundamental importance. Ammonium concentration in the reactor affects the conversion of the nitritation reactor because it may provide either ammonium or oxygen limiting conditions (Harremöes, 1978; Çeçen and Gönenç, 1995; Jianlong and Ning, 2004; Bernet et al., 2005; Pérez et al., 2005; Sliekers et al., 2005; Bougard et al., 2006; Guo et al., 2009; Bartrolí et al., 2010, 2011; Brockmann and Morgenroth, 2010; among many others). A second aspect to take into account is the potential inhibition of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) by free ammonia (FA). Moreover, the following treatment step for complete nitrogen removal via denitrification or anaerobic ammonium oxidation will also benefit from a constant and suitable ammonium concentration in the effluent of the nitritation reactor. What to do to maintain a constant ammonium concentration in the effluent of a nitritation reactor? A basic control strategy to maintain a variable close to a desired reference (known as setpoint value) is to measure it on-line and subsequently apply an action as a function of the difference between the measured value (in this case the ammonium concentration) and the setpoint. This is the basis of a feedback closed-loop control (see Figure 4.1A for a block diagram showing the concept). On-line measurement of ammonium concentration is available at a reasonable cost, and in fact, it has been implemented at laboratory and pilot scale nitritation reactors (Bernet et al., 2005; Bougard et al., 2006; Bartrolí et al., 2010; Li et al., 2011). Indeed ammonium concentration is listed among the commonly used measurements performed by instrumentation on full scale wastewater treatment plants (WWTP's) (Olsson, 2012). However, in full-scale installations of SHARON-Anammox process, the loading rate applied to the nitritation reactor was carefully balanced with the available conversion capacity in the Anammox reactor during start-up (van der Star et al., 2007). From a practical point of view, this is often solved with daily off-line measurements of ammonium, nitrite and nitrate concentrations (van der Star et al., 2007). Therefore, full-scale installations will also benefit from an on-line measurement of the ammonium concentration.

When nitritation is applied to the reject water (a rich ammonium effluent produced in the dewatering of sludge after the anaerobic treatment) the alkalinity is generally adequate to only oxidize 50% of the ammonium (van der Star et al., 2007), actuating implicitly as a regulation of the desired ammonium concentration. However, it is known that operating conditions of the sludge digester, such as the hydraulic residence time, may result in a disturbance of the bicarbonate to ammonium molar ratio, affecting the nitrite:ammonium ratio of the effluent of the nitritation reactor (van Hulle et al., 2010). Consequently, in the SHARON process, the on-line measurement of nitrite:ammonium ratio is considered as essential to gain the desired stability of the subsequent Anammox process (Volcke et al., 2006, 2007).

The indirect control of the ammonium concentration through the on-line measurement of oxygen uptake rate was shown to be crucial for the total and stable washout of NOB when using an activated sludge reactor plus settler configuration for nitritation (Jubany et al., 2009). Similarly, maintaining a high ammonium concentration in the bulk liquid when selecting high activity nitrifiers to enhance nitritation has been reported to be very useful (Chen et al., 2010).

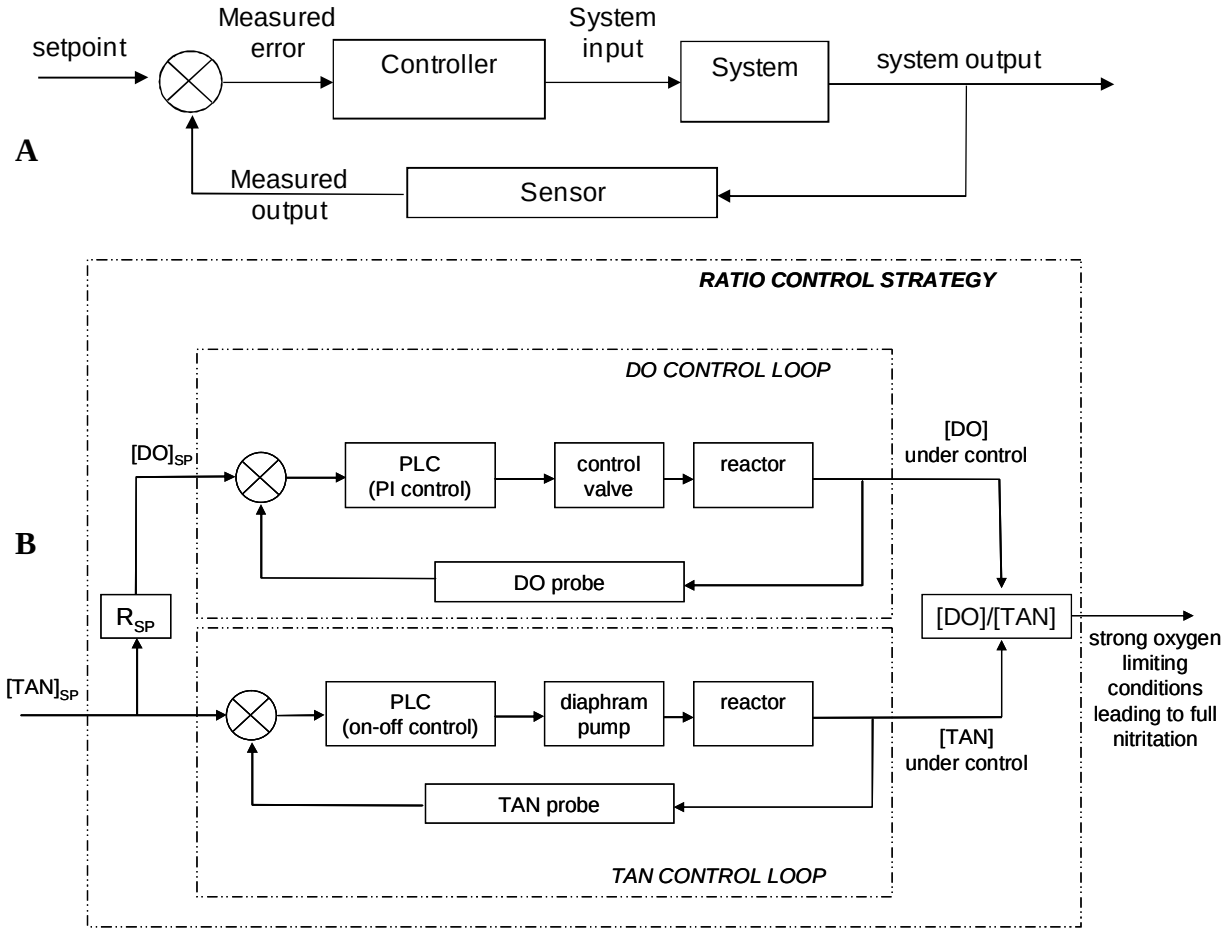

Figure 4.1 (A) Block diagram of a feedback closed-loop control (Stephanopoulos, 1984). (B) Block diagram of the ratio control strategy to obtain full nitritation in aerobic granular sludge reactors operating in continuous mode (Bartrolí et al., 2010). DO: dissolved oxygen; TAN: total ammonia nitrogen. SP: setpoint. R_{SP}: proportionality constant of the ratio station.

When biofilm reactors have been used for nitritation, the control of ammonium concentration in the effluent has been recognized to be a key variable for the start-up and stability of the process (Bernet et al., 2005, Bougard et al., 2006, Bartrolí et al., 2010). When using sequencing batch reactor operation for nitritation, the ammonium concentration in the effluent has been also identified as a very important condition governing the outcomes of the reactor (Ganigué et al., 2012) and in fact, the in-line measurement of ammonium was used to maintain stable nitritation in the long term (Li et al., 2011).

From the engineering point of view, the control of the ammonium concentration in nitritation reactors (which implicitly means the control of the nitrogen loading rate), presents several interesting advantages, given that:

- Prevents the inhibition of AOB by FA during start-up (Jubany et al., 2009).
- Regulates the inhibition of NOB by FA to achieve a stable wash-out of NOB from activated sludge reactors with biomass retention (Jubany et al., 2009).
- Allows for adequate Anammox feeding (Volcke et al., 2006, 2007).

- Maximizes the reactor capacity (Jubany et al., 2009; Bartrolí et al., 2010).
- Assures oxygen limiting conditions required to obtain stable nitritation with biofilm reactors under ratio control strategies (Bernet et al., 2005; Bougard et al., 2006; Bartrolí et al., 2010).

When investigating through modeling the conditions in which nitritation is stable, keeping under control the ammonium concentration in the effluent would be also desirable, since the potential inhibition of AOB and NOB by FA will be easily handled, allowing for straightforward comparison of different operating conditions.

A lack of control of TAN concentration in the effluent has often been a drawback to assess through modeling the appropriate operating conditions required to achieve partial nitrification. For instance, Brockmann and Morgenroth (2010) and Pérez et al. (2009) required large sets of simulations to assess the favorable conditions to achieve nitritation under stable operating conditions. Therefore, a mathematical model able to describe the closed-loop control of ammonium concentration in the effluent will provide an easy assessment of conditions for partial nitrification in biofilm reactors, and additionally, will be an excellent way of investigating the effect of certain key variables independently, because the overlapped effect of several parameters is rather minimized.

This contribution aims to show how the control of the ammonium concentration in the effluent can be implemented in the modeling of granular reactors for nitritation operating in continuous mode. The mathematical model in this type of reactor was validated with the main experimental findings in Bartrolí et al. (2010), including the automatic control of the TAN concentration in the reactor effluent as a closed-loop integrated in a ratio control strategy. Additionally new experimental results were presented, showing the feasibility of the nitritation at 20 ºC. The model will be used to investigate the effects of granule size and temperature on the nitritation process with an aerobic granular reactor operating in continuous mode. The importance of the closed-loop control of TAN concentration will be highlighted through simulations.

4.2 Materials and methods

4.2.1 Reactor set-up and wastewater

A biofilm airlift reactor with working volume of 112 L was used in this study. Compressed air was supplied through an air diffuser placed at the bottom of the reactor. The dissolved oxygen (DO) concentration in the bulk liquid was measured by means of an on-line electrode (LDO sc, HACH LANGE, Düsseldorf, Germany), and a closed feedback control loop was implemented to maintain the DO concentration (see Fig. 4.1B for a detailed description of the block diagram) at different setpoint values (the setpoints applied in each period of operation are detailed in Table 4.2). The controller used was a proportional-integral (PI) controller (see Fig. 4.1B). For the temperature control the reactor was equipped with an electric heating device and a cooling system. The pH control loop used solid $NaHCO_3$ to maintain the pH at 8.2 and to supply the alkalinity required for nitrification. The TAN concentration in the bulk liquid was determined with an on-line probe (NH4D sc probe with a Cartrical cartridge, Hach Lange, Düsseldorf, Germany). A feedback closed-loop control was implemented to maintain TAN concentration at a desired setpoint (see Fig. 4.1B for a detailed version of the block diagram). Different setpoints were applied in each period of operation, as

detailed in Table 4.2. A simple on-off controller was used (see Fig. 4.1B). For further details about the reactor design, instrumentation and control strategy please see Bartrolí et al. (2010).

The biofilm airlift reactor was inoculated with activated sludge from a municipal WWTP (25 L from the main biological reactor at Manresa municipal WWTP, Barcelona, Spain). The reactor was also loaded with 2.5 kg of activated carbon (AC) to induce granulation (Yu et al., 1999). The AC particles used had a mean diameter of 1.2 mm and a wet density of 1.25 g mL^{-1}. After the development of the granular biomass (after about 100 days of operation), the AC particles initially added were removed. The main characteristics of the granular biomass are detailed for each one of the periods of operation in Tables 4.1 and 4.2. Sludge volumetric index ratio at five and thirty minutes (SVI_5/SVI_{30}), had only minor variations in the range 1.00-1.03; and the settling velocity, was in the range [35-57] m h^{-1}. Note how experimental results obtained at 30 ºC were previously reported in the literature (Bartrolí et al., 2010), and the results at 20 ºC are reported for the first time in this contribution.

The biofilm airlift reactor was fed with synthetic wastewater with 4.6 g L^{-1} NH_4Cl (1.2 g of $N\text{-}NH_4^+$ L^{-1}) and the following compounds (in mg L^{-1}): CH_3COONa, 48.0; $CaCl_2 \cdot 2H_2O$, 3.0; KH_2PO_4, 13.0; NaCl, 9.0; $MgCl_2 \cdot 7H_2O$, 6.0; $FeSO_4 \cdot 7H_2O$, 0.13; $MnSO_4 \cdot H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.13; $CuSO_4 \cdot 5H_2O$, 0.07; and H_3BO_3, 0.007.

Table 4.1 Model validation. Comparison of experimental results ("exp.") in steady state obtained applying the ratio control strategy and model outputs ("model"). Experimental results A-E were previously published in Bartrolí et al. (2010). Note that R_{SP} is the proportionality constant of the ratio control strategy (see section 4.3.2 for further details). TAN= total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$); TNN= total nitrite nitrogen (TNN = $N\text{-}NO_2^-$+$N\text{-}HNO_2$).

Period	Temperature (ºC)	R_{SP} (mg O_2 mg^{-1}TAN)	TAN converted to TNN (%)		TAN converted to nitrate (%)		Flow-rate (L day^{-1})	
			exp.	**model**	exp.	**model**	exp.	**model**
A	30	0.17	96	**98**	1	**0**	61	**93**
B	30	0.35	1	**4**	98	**94**	93	**80**
C	30	0.25	98	**98**	1	**0**	79	**74**
D	30	0.18	98	**97**	1	**0**	93	**117**
E	30	0.18	98	**97**	1	**0**	570	**516**
F	20	0.15	98	**98**	1	**0**	56	**60**

Table 4.2 Experimental characterization and reactor conditions for each steady state considered in Table 4.1. All these values were used as inputs for the model in the simulations. The pH was maintained at 8.2 in all periods. Note that $[DO]_{SP}$ and $[TAN]_{SP}$ are the setpoints imposed for DO and TAN concentration in the bulk liquid. DO = dissolved oxygen; TAN= total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$). A-E were previously published in Bartrolí et al. (2010).

Period	Temperature (ºC)	$[DO]_{SP}$ (mg O_2 L^{-1})	$[TAN]_{SP}$ (mg N L^{-1})	Size (mm)	Biomass concentration (g VS L^{-1})	Granule density (g VS $L_{particle}^{-1}$)
A	30	5	30	0.9	0.6	38
B	30	7	20	0.7	0.6	73
C	30	5	20	0.9	0.6	38
D	30	7	40	0.7	0.6	64
E	30	7	40	0.7	4.6	67
F	20	4.5	30	0.7	0.8	40

4.2.2 Analytical methods

Regular sampling of the bulk liquid from the pilot plant was carried out to determine the TAN, total nitrite nitrogen (TNN = $N\text{-}NO_2^-$ + $N\text{-}HNO_2$), and nitrate concentrations through off-line analysis. The TAN concentration in off-line liquid samples withdrawn from the bulk liquid was determined by means of a continuous flow analyzer. The TNN and nitrate concentrations were measured by ion chromatography (ICS-2000 Integrated Reagent-Free IC System, DIONEX). Volatile solids (VS), total solids (TS), and SVI were determined according to Standard Methods (APHA, 1995). The granular biomass was characterized in terms of size and granule density. 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 analysis was carried out using MIL-Lite Matrox Inspector 3.1. The procedure followed was (i) to convert the original image of granules to black and white mode for the image processing, (ii) to define the threshold corresponding to the area of interest in the image, i.e. the granules and (iii) to export the selected data with the software (e.g., area, perimeter, roundness, average diameter, etc.) to a worksheet. For each determination at least 70 granules were used. The density of the granules biomass was measured using the Blue Dextran method described by Beun et al. (2002).

4.3 Model description

4.3.1 Biofilm model, kinetics and parameters

A one-dimensional biofilm model was developed to simulate the nitrifying biofilm airlift reactor performance based on Wanner and Reichert (1996) and implemented in the software package AQUASIM (Reichert, 1998), v.2.1d. The reactor volume was assumed to be fixed at 112 L.

The biomass species described as particulate compounds in the biofilm matrix were four: ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), heterotrophic bacteria and inert biomass. Biofilm area was described as a function of the granule radius, to correctly simulate the biofilm geometry (for further details see below Eq. 4.5). Total biofilm area was defined as a function of granule size and number of granules. A detachment rate was used to keep a constant biofilm thickness in steady state at a predefined value. Detached biomass from the biofilm was considered as active following the same kinetics defined for the biomass in the biofilm. Attachment of biomass onto the biofilm surface has been neglected. For the sake of simplicity external mass transfer has been neglected. The porosity of the biofilm was fixed as 80% and kept constant during all the simulations. Initial fractions of particulate compounds were 10% AOB, 8% NOB and 2% heterotrophic biomass. The microbial kinetics and the stoichiometry used are detailed in the Appendix A (Tables A1-A3 in supplementary materials). Growth of AOB and NOB included inhibition by free ammonia (FA) and free nitrous acid (FNA) as proposed by Jubany et al. (2008). Decay of AOB and NOB was described as recently proposed by Munz et al. (2011), with a single rate equation which includes two decay coefficients: a decay coefficient for aerobic conditions and another one for anoxic / anaerobic environments, which likely will occur in the biofilm depth. Other parameters related to the biofilm and used in the model are detailed in Table A4 (in Appendix A).

4.3.2 Modeling the TAN control loop inside the ratio control strategy

One of the key aspects of the development of the mathematical model was to provide a powerful approach able to simulate the ratio control strategy (see Fig. 4.1B for a conventional block diagram). The control strategy has two different closed-loops: (i) one to maintain the TAN concentration in the bulk liquid (i.e., the reactor effluent, considering a well-mixed liquid phase in the reactor) and (ii) a second one to control the DO concentration in the bulk liquid. Each one of these two control loops has to be programmed independently and in addition they have to be linked through the proportionality constant (R_{SP}) to become a true ratio control strategy (Hägglund, 2001; Bartroli et al., 2010).

For the mathematical description of the DO control loop, aeration was introduced as a dynamic process only active in the bulk liquid phase. A high value for the volumetric gas-liquid oxygen transfer coefficient ($k_La = 10^4$ d^{-1}) was selected. The oxygen solubility used was equal to the DO setpoint (Pérez et al., 2009):

$$\frac{d[DO]}{dt} = k_L a \cdot ([DO]_{SP} - [DO]) \tag{4.1}$$

Where [DO] is the dissolved oxygen concentration in the bulk liquid, and $[DO]_{SP}$ is the DO concentration setpoint.

For the TAN control loop, an *ad hoc* expression was developed, because the control loop has the inflow rate (Q_{in}) as manipulated variable:

$$Q_{in} = Q_{in,0} \cdot \left(1 + \frac{[TAN]_{SP} - [TAN]}{[TAN]_{SP}} \cdot a\right) \tag{4.2}$$

Where $Q_{in,0}$ is known as the bias of the control action, i.e. the default value of flow-rate. The controller will always act either increasing or decreasing Q_{in} around $Q_{in,0}$. [TAN] is the TAN concentration in the bulk liquid phase. $[TAN]_{SP}$ is the TAN concentration setpoint. *a* is the proportional gain of the controller, easily tuned in each simulation depending on the particular operating conditions. The principle of the performance of the expression is similar to that applied in a conventional proportional control law. The action will be stronger when the measured value of TAN concentration is far from the setpoint, whereas when TAN concentration is approaching the setpoint, the action of the controller will be weaker.

The setpoints were linked through the proportionality constant (R_{SP}) as $[DO]_{SP}=R_{SP}\cdot[TAN]_{SP}$.

Experimental studies found in the literature with nitrifying biofilm reactors use either (i) high values of R_{SP}, being TAN the limiting substrate, and therefore complete nitrification is achieved ($R_{SP} \geq 1$ mg O_2 mg^{-1} TAN, for instance Jih et al., 2008), or (ii) very low values of R_{SP}, which imposed a very strong DO limitation, leading to important nitrite build up ($0.02 \geq R_{SP} \geq 0.01$ mg O_2 mg^{-1} TAN, Tokutomi, 2004). Nevertheless, there is a clear lack of information related to intermediate R_{SP} values. In that intermediate range corresponding to moderate R_{SP} values ([0.1-0.5] mg O_2 mg^{-1} TAN), a nitrifying granular reactor will switch from nitrification to full nitritation with only a slight variation of the setpoints ratio (R_{SP}) (Bartrolí et al., 2010), allowing for an easy assessment of the effect of key operating conditions.

4.3.3 Simulation strategy for model validation

The steady states experimentally obtained in Bartrolí et al. (2010) were considered for the validation of the mathematical model, as shown in Table 4.1. Operational conditions and some key experimental measurements were used as inputs for the model in each one of the steady states considered (see Table 4.2). In particular, experimental values of biomass concentration (X_{exp}) and biofilm density (rho_{exp}) for each period (A-F in Table 4.2) were used to estimate the total biofilm volume in the reactor ($V_{biofilm}$):

$$V_{biofilm} = X_{\exp}\cdot V_{reactor}\cdot\frac{1}{rho_{\exp}} \tag{4.3}$$

Where $V_{reactor}$ stands for the total reactor volume. Using this estimation of the biofilm volume, the number of granules (N_{Gr}) could be calculated by assuming a mean granule size (D_{exp}, experimentally measured for each period of operation, see Table 4.2):

$$N_{Gr} = \frac{V_{biofilm}}{\frac{4}{3}\pi\left(\frac{D_{\exp}}{2}\right)^3} \tag{4.4}$$

Therefore, the biofilm surface area (*A*) is introduced in AQUASIM through the expression:

$$A = N_{Gr}\cdot 4\pi z^2 \tag{4.5}$$

Where *z* stands for the radial direction of biofilm growth (declared as a program variable in AQUASIM).

Steady state granule size was imposed to be equal to the experimental one. To assure steady state in the simulations, they were run for a period of operation of 15,000 days, and results in terms of biofilm (biofilm thickness and biomass fractions in the biofilm depth) and N compounds concentrations in the bulk liquid were inspected to check that constant values were achieved. The main outputs of the model were (at steady state): inflow rate (as a result of the TAN control loop described in Eq.(4.2)), nitrite and nitrate concentrations in the effluent (see Table 4.1 for a direct comparison to the experimental values in Bartrolí et al., 2010).

4.3.4 Scenarios to test the importance of the control of ammonium concentration

To show the importance of controlling the TAN concentration in biofilm reactors used for nitritation, two different scenarios were explored in which possible variations of the TAN concentration in the reactor may result in periodic variations of DO/TAN concentration ratio, potentially leading to nitrate production.

Scenario 1. Poor control on a monthly basis: First a R_{SP} = 5/23 = 0.22 mg O_2 mg^{-1} TAN was applied until nitritation was attained in steady state. Then the periodic disturbance was applied: during one third of the month the TAN concentration was decreased to 5 mg N L^{-1} (while keeping DO constant at 5 mg O_2 L^{-1}, resulting in a DO/TAN concentration ratio of one) and this disturbance pattern was repeated during 400 days, and then relaxed back to the previous R_{SP} value (0.22 mg O_2 mg^{-1} TAN). Additionally, to get a more realistic simulation, 10% random variations of the inflow TAN concentration were applied, to show how the control loop handle those changes.

Scenario 2. Poor control on a daily basis: when an automatic control loop was not implemented for ammonium control a daily off-line measurement of the TAN concentration was sometimes used to apply corrective changes on a daily basis in the loading rate as already mentioned in the introduction (van der Star et al., 2007). To explore the effects of applying corrective changes in the loading rate, a simulation was defined in which daily changes of TAN setpoint are applied: during one third of the day the TAN concentration was decreased to 5 mg N L^{-1} and this disturbance pattern was repeated during 400 days, and then relaxed back to the previous R_{SP} value (0.22 mg O_2 mg^{-1} TAN), similarly to the previous scenario.

4.4 Results and discussion

4.4.1 Model validation

For model validation a set of experimental results in steady state previously published in Bartrolí et al. (2010) were considered and they were detailed in Tables 4.1 and 4.2. Additionally, the new experimental results obtained at 20ºC were also considered for the validation, but they are treated in a separate section (see section 4.4.5). The model was able to predict when full nitritation is maintained at steady state for all experimental periods at 30ºC (A-E, see Table 4.1 for a direct comparison of model predictions and experimental results). Note how the modeling results show a good agreement with experimental results when imposing different TAN and DO setpoints, at different

biomass concentrations and with different granules sizes (see details in Table 4.2). In period B, complete nitrification was observed experimentally. The model output predicted a high nitrate production, with a slight nitrite accumulation (48 mg N L^{-1}, corresponding to 4% of TAN converted to TNN, see Table 4.1, period B). Experimentally, the maximum value of R_{SP} that could lead to full nitritation in steady state (denoted as $R_{max,fN}$) was narrowed within [0.25-0.35] mg O_2 mg^{-1} TAN (Bartrolí et al., 2010), and this same range was found through simulation.

Another output of the model was the inflow rate. Direct comparison of the experimental inflow rate with that obtained in the simulations is also presented in Table 4.1. The mathematical model was able to predict the trend although there were some deviations. Notably, prediction is relatively accurate even for period E, in which a very high flow-rate was experimentally achieved.

4.4.2 Highlighting the effects of the closed-loop control

When the DO/TAN concentration ratio was decreased periodically during one third of a month (scenario 1), a fast nitrate built-up was obtained after two months, and in fact, after less than 100 days, a nitrate-rich effluent was produced as predicted by the model (see Fig. 4.2). After relaxation of the periodic disturbances, the reactor was able to produce again full nitritation (see Fig. 4.2A). If in addition to the TAN concentration disturbances, DO was manipulated to keep a constant value for the DO/TAN concentration ratio in the reactor, full nitritation was always obtained showing the efficiency of the ratio control strategy (Fig. A2 in Appendix A). To assess the effect of inhibition by free ammonia, the simulation was run again but considering that there was no inhibition of AOB and NOB by FA ($K_{I,TAN,AOB} = K_{I,TAN,NOB} = 10^4$ mg N L^{-1}). Very similar results were obtained because full nitritation was lost during the periodic disturbances (see a zoomed in graph in Fig. 4.3), demonstrating that the effect of (strong) DO limitation overrides the effect of inhibition in this reactor type in agreement with previous theoretical and experimental research (Pérez et al., 2009; Bartrolí et al., 2010; Brockmann and Morgenroth, 2010).

Similarly, for periodic disturbances applied on a daily basis (scenario 2), the results are presented in Fig. 4.4. The shorter the time scale of the periodic disturbance the less nitrite was accumulated in the reactor. When comparing the results of the simulation with those obtained including inhibition of AOB and NOB by FA (see a zoomed in graph in Fig. 4.4B), smaller differences in the nitrite accumulation were obtained in the second scenario. If in addition to the TAN concentration disturbances, DO was manipulated to keep a constant value for the DO/TAN concentration ratio in the reactor, full nitritation was always obtained, demonstrating the efficiency of the ratio control strategy (Fig. A3 in Appendix A).

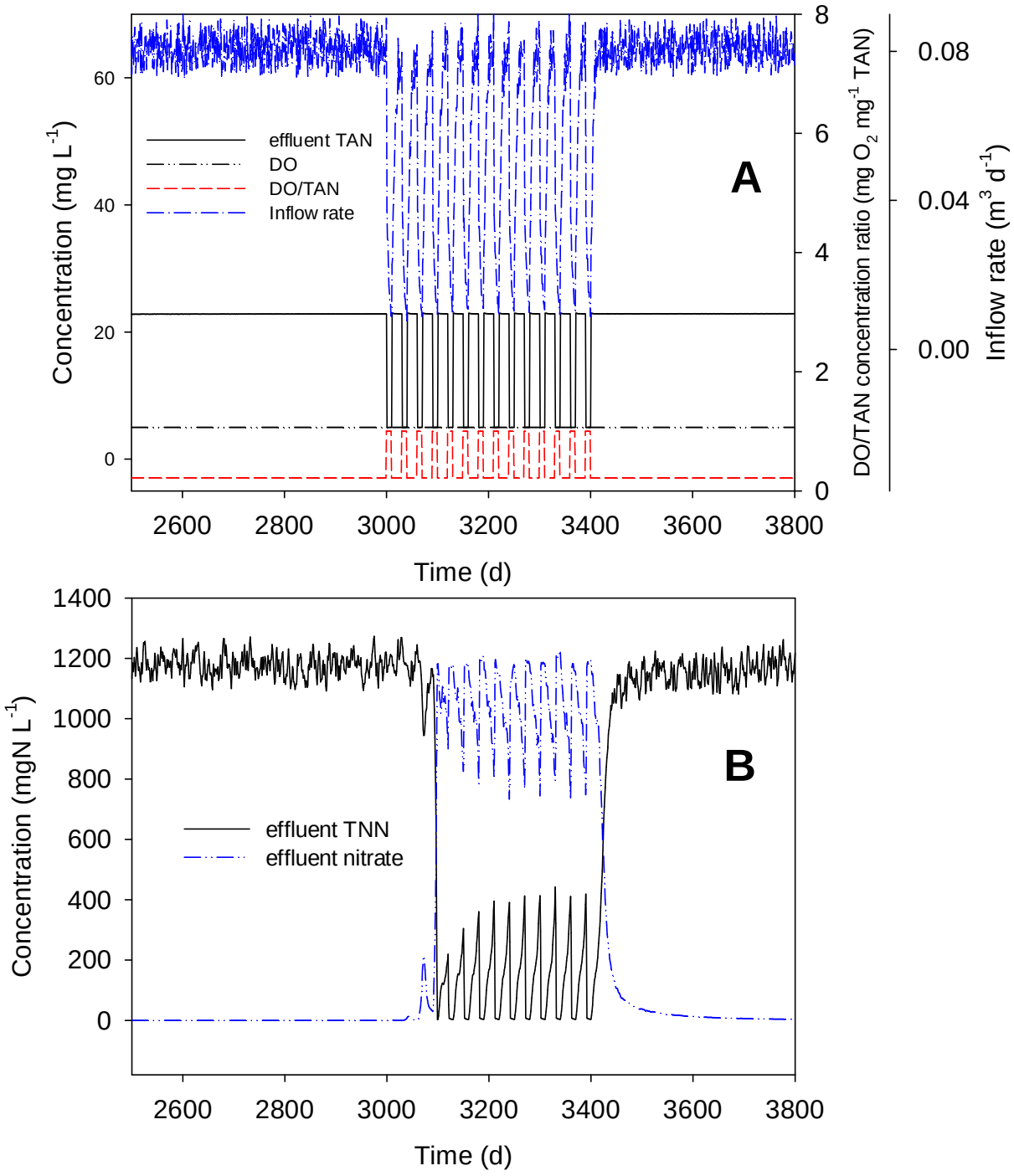

Figure 4.2 Poor control in a monthly basis (scenario 1). TAN concetration decreased from 23 to 5 mg N L^{-1} periodically (one third of the month at low TAN concentration) during 400 days. Note how DO concentration was kept constant at 5 mgO_2 L^{-1}. All data plotted correspond to modeling results.

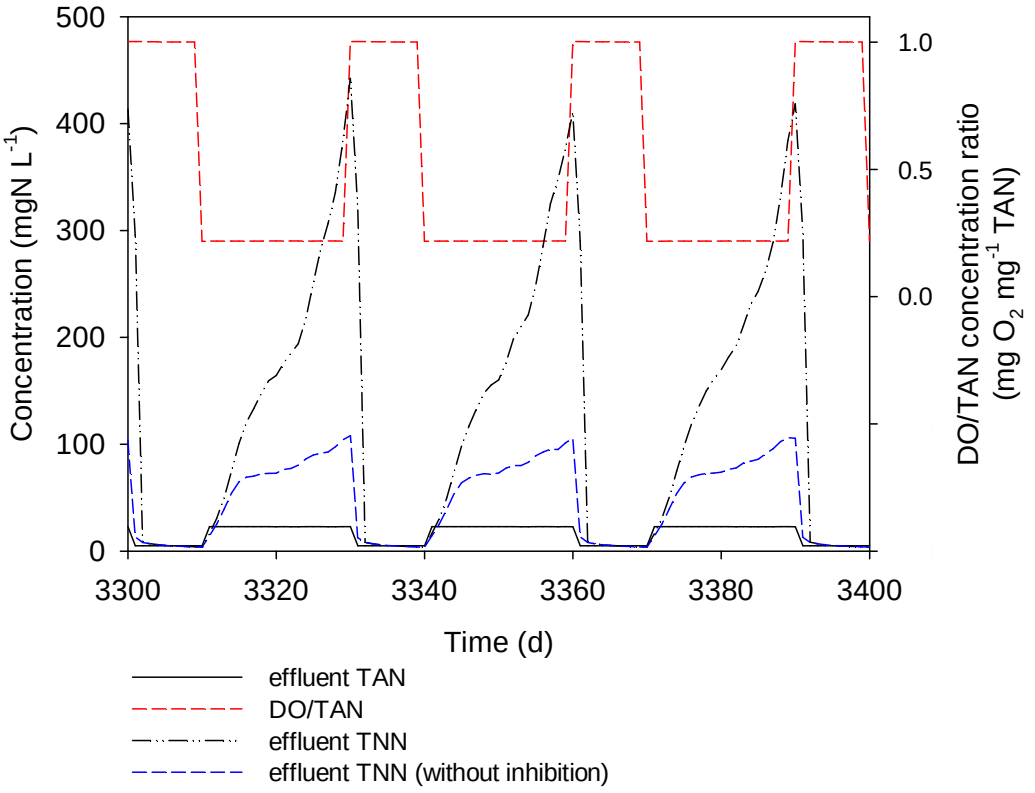

Figure 4.3 Zoommed in graph of Fig. 4.2B to show the effect of the DO/TAN ratio on nitritation. The simulation was also computed considering no inhibition of AOB and NOB by free ammonia ($K_{I,TAN,AOB} = K_{I,TAN,NOB} = 10^4$ mg N L^{-1}). DO concentration was kept constant at 5 mgO_2 L^{-1}. All data plotted correspond to modeling results.

4.4.3 Effect of the DO/TAN concentration ratio on the applied loading rate

Experimental conditions in period C (see Tables 4.1 and 4.2) were used to explore how the DO/TAN concentration ratio affects the applied volumetric nitrogen loading rate (NLR_v) for a wide range of values. The results obtained with this set of simulations are presented in Fig. 4.5. The specific setpoints considered in the simulations presented in Fig. 4.5 are detailed in Appendix A (Table A5). Note how although only setpoints are plotted, the efficiency of the control allows to consider for practical purposes that setpoint and actual values are the same (for examples see Table A5 in Appendix A). The results presented in Fig. 4.5A or 4.5B show the procedure to determine the maximum value of the DO/TAN concentration ratio leading to full nitritation (i.e. $R_{max,fN}$). $R_{max,fN}$ will define the limit of the domain in which the reactor can be operated with stable full nitritation.

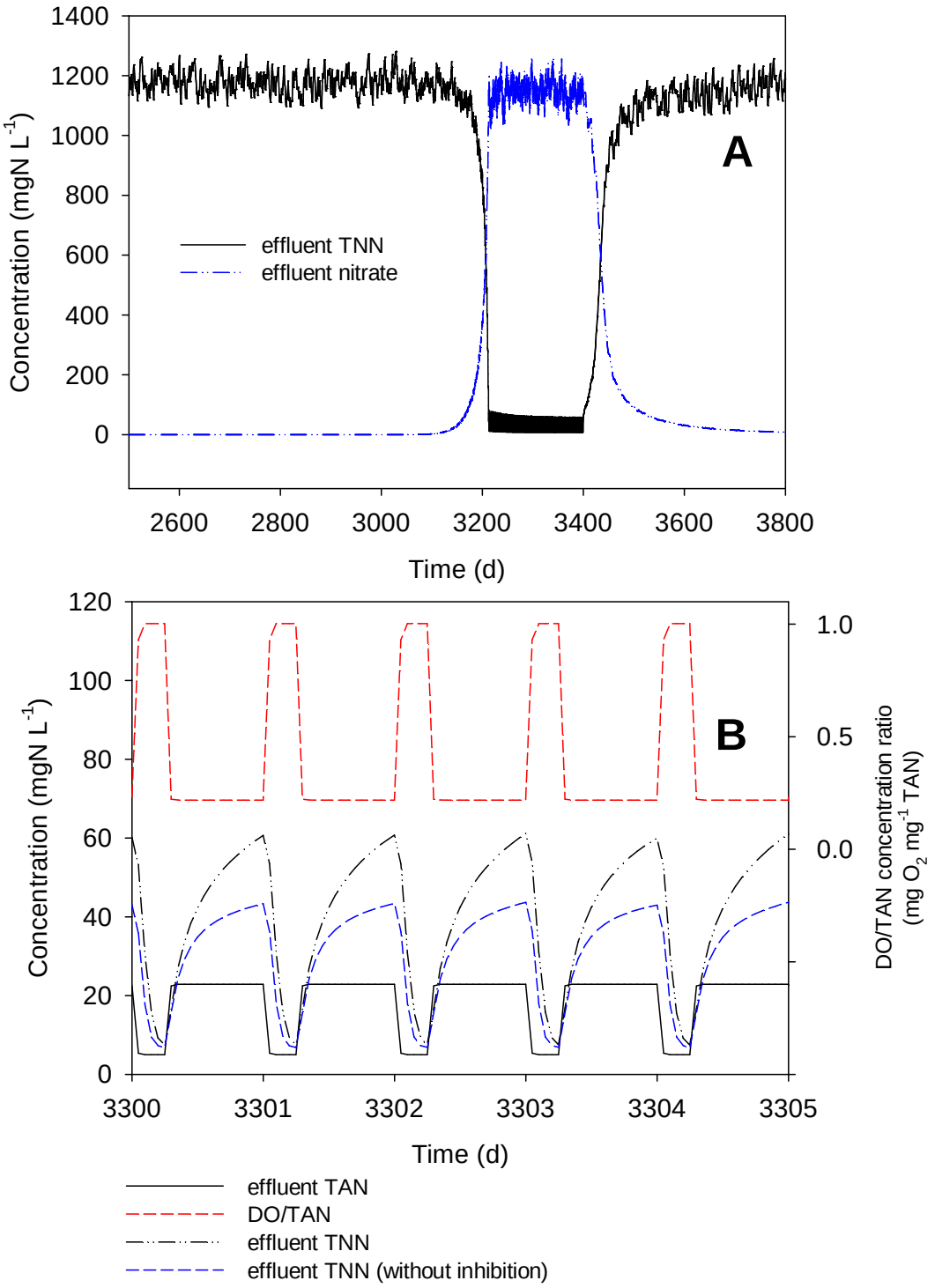

Figure 4.4 Poor control in a daily basis (scenario 2). TAN concentration decreased from 23 to 5 mg N L^{-1} periodically (one third of the day at low TAN concentration) during 400 days. (B): zommed in graph of Fig. 4.4A to show the effect of the DO/TAN ratio on nitritation. The simulation was also computed considering no inhibition of AOB and NOB by free ammonia ($K_{I,TAN,AOB}$ = $K_{I,TAN,NOB}$ = 10^4 mg N L^{-1}). DO concentration was kept constant at 5 mgO_2 L^{-1}. All data plotted correspond to modeling results.

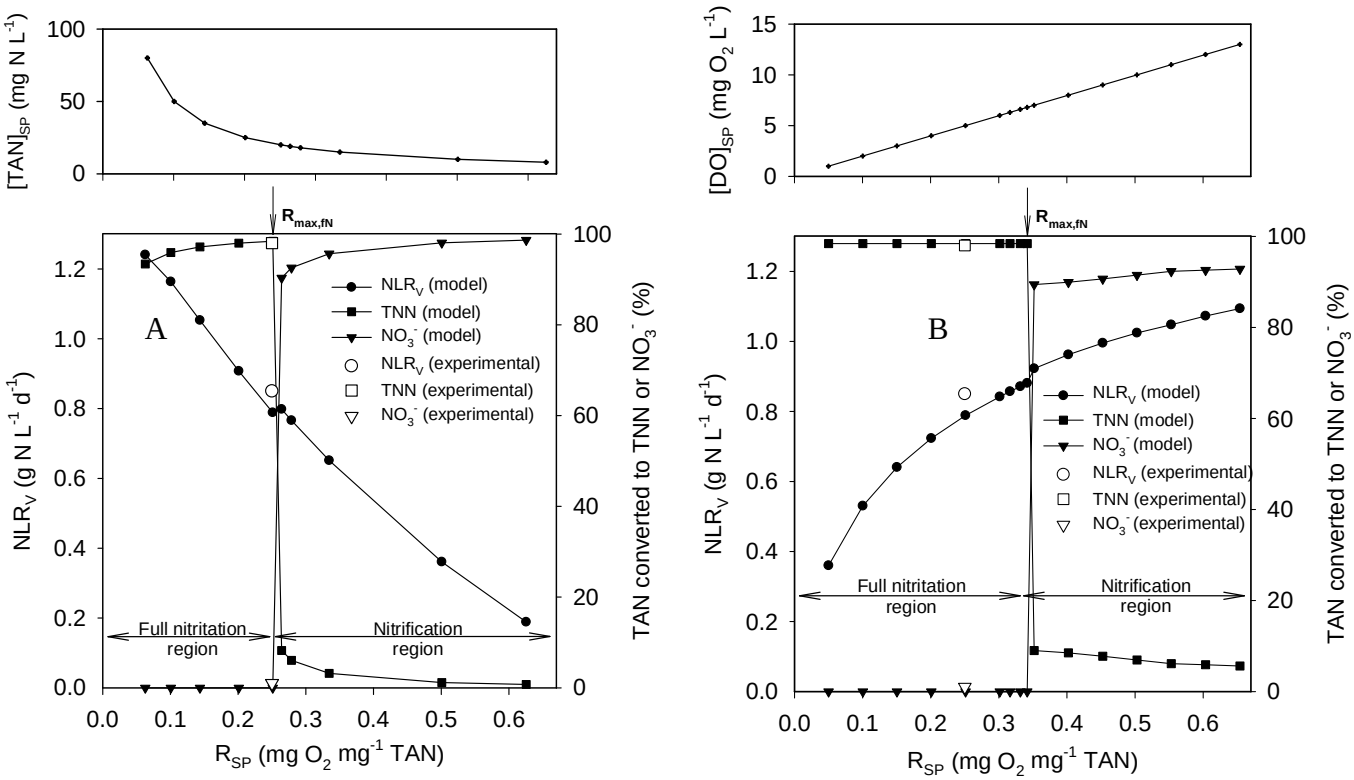

Figure 4.5 Steady state conversions and volumetric nitrogen loading rate applied (NLR_v) for different proportionality constants (R_{SP}) used by the ratio control strategy as simulated with the mathematical model (temperature 30 ^oC, granule size of 0.9 mm). The maximum value of R_{SP} leading to full nitritation ($R_{max,fN}$) was indicated by a vertical arrow. Experimental results for steady state C (Table 4.1) regarding conversion and NLR_v were plotted with void symbols for a direct comparison. The rest of (filled) symbols correspond to modeling results. (A): simulated results were obtained for a constant dissolved oxygen concentration setpoint: $[DO]_{SP}$=5 mg O_2 L^{-1}, and manipulating total ammonia nitrogen concentration setpoint ($[TAN]_{SP}$) . (B): simulated results were obtained for a constant total ammonia nitrogen concentration setpoint: $[TAN]_{SP}$ = 20 mg N L^{-1}, and manipulating dissolved oxygen concentration setpoint ($[DO]_{SP}$).

Two different sets of simulations were performed. In the first one, $[TAN]_{SP}$ was the manipulated setpoint whereas $[DO]_{SP}$ was always kept constant at 5 mg O_2 L^{-1} (Fig. 4.5A). In Fig. 4.5A two very different regions could be distinguished. For values of $R_{SP} < R_{max,fN}$ full nitritation was always found at steady state, with negligible conversion to nitrate. But for values of $R_{SP} > R_{max,fN}$ the reactor effluent had an important concentration of nitrate, i.e. approaching to complete nitrification. The $R_{max,fN}$ was found to be equal to 0.26 mg O_2 mg^{-1} TAN. At each value of R_{SP} explored, a different value for the inflow rate was achieved in steady state, and consequently a different value for NLR_V was found. This is due to the action of the TAN control loop, since to keep the desired $[TAN]_{SP}$ the inflow rate was regulated, as already explained. The higher the $[TAN]_{SP}$ used, the higher the NLR_v that could be achieved in steady state. The main cause of this negative slope of the NLR_v curve is that at a higher $[TAN]_{SP}$ the rate of substrate removal strongly increases because of a higher growth rate of AOB (the

Monod term $\frac{[TAN]}{[TAN]+K_{S,TAN}}$ will be closer to unity, see the rate equation in Table A2 in Appendix A).

On the other hand, a second way of screening the effect of R_{SP} on the applied loading rate is by maintaining a constant value for the $[TAN]_{SP}$ and manipulating the $[DO]_{SP}$ (Fig. 4.5B). Two regions could also be distinguished in Fig. 4.5B, as already explained for Fig. 4.5A. The breaking point separating both regions was found to occur at a higher R_{SP} value, $R_{max,fN}$ = 0.35 mg O_2 mg^{-1} TAN. This difference in the $R_{max,fN}$ found with both methodologies demonstrates that the absolute value for each setpoint ($[TAN]_{SP}$ and $[DO]_{SP}$) has a slightly different impact. Consequently, the DO/TAN concentration ratio is not always enough to fine-tune the appropriate R_{SP} to achieve and maintain full nitritation.

The NLR_v curve in Fig. 4.5B has a positive slope. Operating at higher R_{SP} implies higher $[DO]_{SP}$. Since strong oxygen limiting conditions were applied in the reactor, imposing a higher $[DO]_{SP}$ increased the oxygen flux towards the biofilm, consequently a higher NLR_v can be treated in the reactor. Note how strong oxygen limiting conditions are possible even at relatively high DO concentrations in the bulk liquid (for detailed reasons about the influence of DO/TAN concentration ratio on oxygen limitation see Bartrolí et al., 2010).

In Fig. 4.5 experimental points obtained for steady state C were also plotted in the same graphs to show the agreement between the model predictions and the experimental results with regard to NLR_v and conversion of TAN to TNN and to nitrate.

4.4.4 Effect of granule size on $R_{max,fN}$

To explore the possible effect of the granule size on the conditions at which full nitritation is feasible, a specific set of simulations was carried out. Using the experimental conditions in steady state C, different simulations maintaining the same total biofilm surface were run. For each selected value of the granule size the number of granules required was calculated to keep a fixed value of the biofilm area. Maintaining a fixed area assures that the resulting values of $R_{max,fN}$ (found with the simulations) will depend only on the mean granule size selected. Since DO limitation will prevail at $R_{max,fN}$, the granule will be partially penetrated by oxygen, only a small granule fraction will be active. Consequently, it has no sense the option of keeping a constant biofilm volume.

An iterative calculation procedure was defined in order to determine the $R_{max,fN}$ value for each granule size. The $[TAN]_{SP}$ was kept constant at 20 mg NL^{-1} in all simulations, whereas $[DO]_{SP}$ was manipulated to find the highest $[DO]_{SP}$ value at which a steady state with only nitrite (and no nitrate) in the effluent is found (i.e., $R_{max,fN}$). This iterative procedure was repeated for a set of different granule sizes in the range [0.05-3] mm. The iterative procedure has been depicted in a flow diagram in Appendix A (Fig. A4). Results are summarized in Fig. 4.6 (see for instance the curve at 30 ºC). The obtained results clearly showed that the $R_{max,fN}$ value can be affected by the granule size. Higher values of $R_{max,fN}$ were found in a range of granule size between 1 and 3 mm. Nevertheless, very low $R_{max,fN}$ values were found for very small granules. A very low

value of $R_{max,fN}$ means that the region in which full nitritation is feasible is very reduced, and requires very low values of $[DO]_{SP}$.

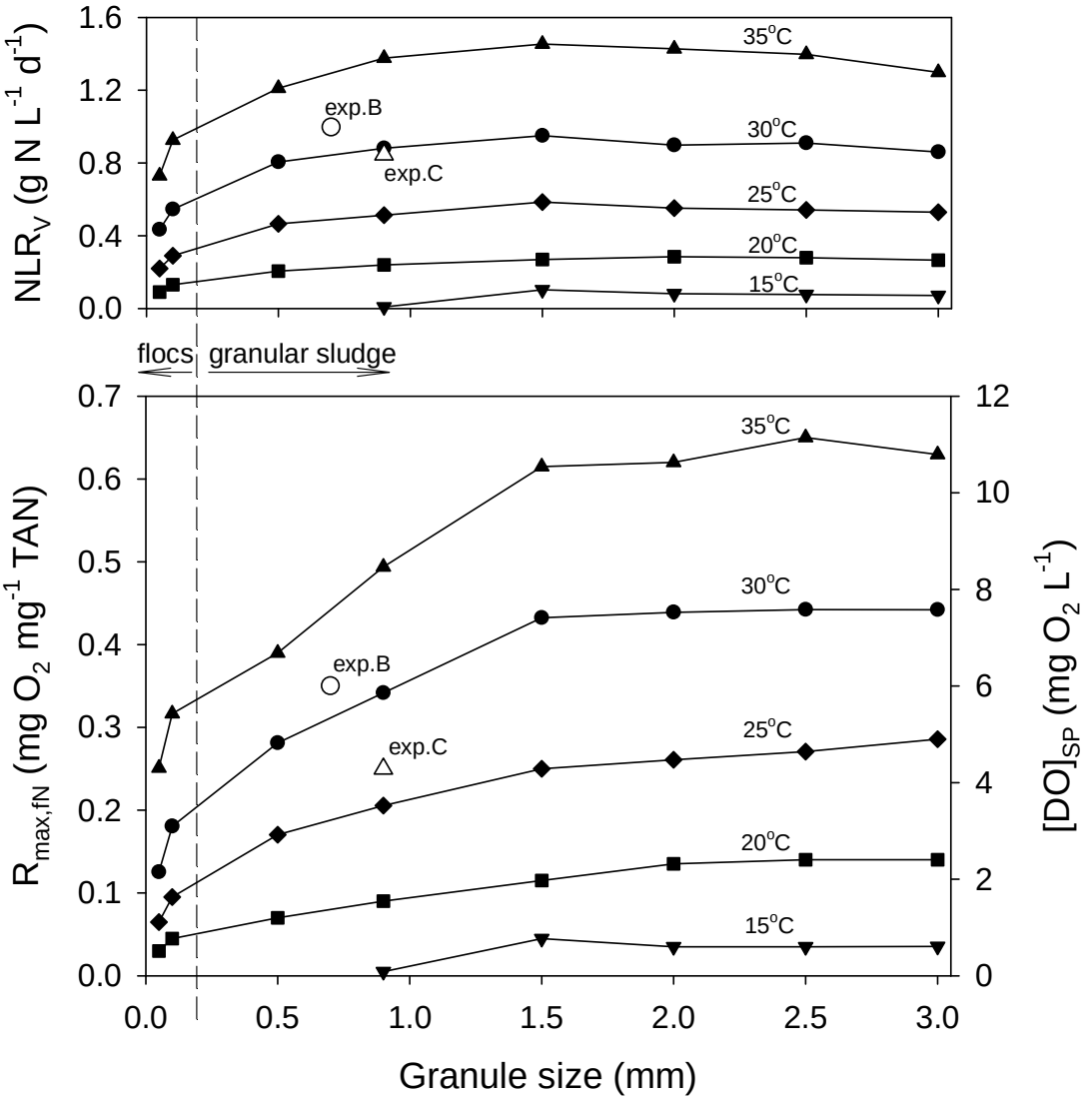

Figure 4.6 Simulated results on the effect of the granule size on (i) the maximum value of setpoints ratio leading to full nitritation ($R_{max,fN}$) and on (ii) the volumetric nitrogen loading rate (NLR_v) at different temperatures of operation. Dissolved oxygen concentration setpoint ($[DO]_{SP}$) has been also added to the graph. Dashed line indicates a size of 0.2 mm which is often considered the minimal size of a granule. Simulated results were obtained for a constant total ammonia nitrogen concentration setpoint: $[TAN]_{SP}$ = 20 mg N L^{-1}, and manipulating $[DO]_{SP}$. Experimental values of R_{SP} for steady states B and C (obtained at 30 ºC) were also plotted with void symbols. The rest of (filled) symbols correspond to modeling results.

For very small granule sizes, the reactor will be very similar to a reactor with cells in suspension (activated sludge), in which a very low DO concentration in the bulk is required to produce a nitrite rich effluent when ammonium is in excess (Sliekers et al., 2005). When the granule size is bigger (for instance between 1 and 3 mm at 30 ºC in Fig. 4.6), strong oxygen gradients appear in the biofilm and ease the conditions for

AOB to outcompete NOB (due to the higher oxygen affinity of the first compared to the latter), and therefore allowing for full nitritation at higher DO concentrations.

In addition, the whole procedure was repeated at different temperatures, see next section for an assessment of the results found.

Experimental results obtained for steady states B and C (both at 30 ºC, see Table 4.2) were also plotted in Fig. 4.6. Note how the experimental values plotted correspond to R_{SP} but not to $R_{max,fN}$. Interestingly, experimental R_{SP} value for steady state B is above the curve corresponding to 30 ºC, showing that complete nitrification was predicted by the model, as found experimentally. Similarly for steady state C, the experimental R_{SP} value used at 30 ºC is below the corresponding curve predicted by the model, thus allowing for full nitritation and therefore showing a good agreement between experimental results and model predictions.

The modeling results presented in Fig. 4.6 also suggest that for granule size higher than 1.5 mm there is no further impact of environmental / substrate conditions, probably due to the diffusion limitation and the existence of high inert fractions in the granule core.

4.4.5 Effect of temperature on $R_{max,fN}$ at different granule sizes

The pilot scale reactor was operated using the ratio control strategy at 20 ºC. Steady state results obtained experimentally are shown in Table 4.1, period F. These experimental results are new and they were not previously reported in the literature.

The ability of the model to describe the experimental results obtained for period F at 20 ºC (see steady state results of the simulation in Table 4.1, period F, "model") was tested. In view of the results, the model was considered able to describe the experimental results at 20 ºC, hence the temperature dependence expressions used for the growth and decay rates are suitable for this particular simulation study. Achievement of partial nitrification in biofilm reactors at low temperature was previously reported by Antileo et al. (2007) with a rotating biological contactor; and theoretically proven through simulations in Wyffels et al. (2004) and Brockmann and Morgenroth (2010).

Using the experimental conditions in steady state C (see Table 4.2) the effect of temperature of operation on the $R_{max,fN}$ value was assessed. For instance, to find $R_{max,fN}$ in Fig. 4.6, for each granule size considered, the $[DO]_{SP}$ was manipulated to find the highest $[DO]_{SP}$ value at which a steady state with only nitrite (and no nitrate) in the effluent is found (i.e., $R_{max,fN}$). Any value of R_{SP} over the curve of $R_{max,fN}$ (i.e., for $R_{SP} > R_{max,fN}$) will lead to a steady state in which both nitrite and nitrate will be present in the effluent of the reactor. On the contrary, any value of R_{SP} below the curve of $R_{max,fN}$ (i.e., for $R_{SP} < R_{max,fN}$) will lead to a steady state in which only nitrite and no nitrate will exit the reactor, i.e., full nitritation.

The study was extended for a wide range of granule sizes whereas total biofilm surface was kept constant as discussed in the previous section (see Table A6 in Appendix A for details). The results clearly indicated that the $R_{max,fN}$ was affected significantly by the temperature of operation, as expected (see Fig. 4.6). At high operating temperature, $R_{max,fN}$ increased considerably, i.e. full nitritation can be maintained at higher DO concentrations. It is easier for AOB to outcompete NOB in the biofilm at higher

temperatures, because of a higher difference of maximum specific growth rates. Model predictions showed how, when the temperature of operation decreased strongly, the range of operating conditions to maintain full nitritation was as well importantly reduced. At 15 ºC the model predicted full nitritation only when granules size was around 1.5-2 mm, and imposing a rather low DO concentration, which would yield as a consequence, a rather low nitrogen loading rate (see Fig. 4.6).

The DO concentrations required to switch from full nitritation to complete nitrification were found to be beyond the saturation value in a wide range of the tested conditions. In these conditions, if only air is used to supply oxygen to the process (which is the habitual choice), full nitritation will always be obtained in the reactors. This is in agreement with experimental results found by Antileo et al. (2007) when using a rotating biological contactor, as well as those reported by Bartrolí et al. (2010) for an airlift biofilm reactor. These researchers found high percent of nitritation at high dissolved oxygen concentrations.

To complete the overall analysis, a more in depth study is required, in which the effect of biomass concentration, biofilm surface area and nitrous and nitric oxide emissions should be considered to correctly assess the selection of the targeted DO concentration. Further research would be desirable on this specific issue.

4.5 Conclusions

- The importance of closed-loop control of ammonium concentration in aerobic granular reactors performing nitritation has been demonstrated using a model based study.
- Granule sizes higher than 1.5mm enhance the possibilities to obtain stable full nitritation.
- The closed-loop control of ammonium concentration as part of a ratio control strategy has proven to produce stable full nitritation even at 20ºC.
- Modeling results estimated that even at 15ºC full nitritation could be achieved.

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Chapter 5

The achievement of high rate nitritation with aerobic granular sludge reactors enhanced by sludge recirculation events

Summary

A ratio control strategy has been used to demonstrate the feasibility of this automatic control procedure for the achievement of stable full and partial nitritation. The control strategy assured constant DO/TAN concentration ratio in the bulk liquid of aerobic granular sludge reactors operating in continuous mode. Three different set-ups with different reactor capacities were used (3, 112 and 150 L). High strength synthetic wastewaters and reject water were tested with similar performance. Achieved nitrogen loading rates ranged between 0.4 and 6.1 kg N m^{-3} d^{-1}, at temperatures between 20 and 30 ºC. Granular sludge and nitritation were stable in the long term continuous operation of the reactors. Suitable stable effluent for Anammox has been obtained using the desired TAN setpoint (i.e. 50% of influent ammonium oxidation). An existing biofilm model developed incorporating the implemented control loops and validated in a previous publication was used to investigate the effects of the ammonium concentration of the influent and the biofilm density on the achievement of full nitritation. The model demonstrated how sludge recirculation events led to a stable and significant increase of the biomass concentration in the reactor, which in turn resulted in the achievement of high nitrogen loading rates, due to the action of the control strategy. The model predicted an enhancement of stable full nitritation at higher ammonium concentrations in the influent. Poor influence of the biofilm density in the achievement of full nitritation was predicted with the model.

5.1 Introduction

Nitrogen removal via nitrite has recently gained increasing interest because of its associated economic savings when compared to classical nitrogen removal technologies, in which the ammonium is fully oxidized to nitrate. Currently, nitritation is applied as an advanced wastewater treatment in municipal wastewater treatment plants (WWTP) for the biological nitrogen removal (BNR) of the reject water, the rich ammonium effluent produced in the dewatering of sludge after the anaerobic treatment. To this effect, different alternatives have been developed, including nitritation plus heterotrophic denitrification, nitritation plus anaerobic ammonia oxidation (Anammox, (Strous et al., 1999)) or even single-step nitritation-Anammox granular reactors (Third et al., 2001).

Biofilm reactors have been regarded as a good alternative to improve the efficiency of nitritation, and several research studies were focussed on this issue (Garrido et al., 1997; Bernet et al., 2005; Bougard et al., 2006). Recently, a ratio control strategy was successfully applied to biofilm airlift reactors to obtain full nitritation (100% ammonia conversion to nitrite) under stable operating conditions, the so-called automatic control for partial nitrification to nitrite in biofilm reactors (ANFIBIO; (Bartroli et al., 2010); see also Fig. 5.1). Major advantages of this type of treatment are high nitrogen loading rates, together with a very robust operation in the long term operation (Bartroli et al., 2010).

We would like to present the major achievements obtained with the ANFIBIO technology using three different sets of results: (i) operation with laboratory and pilot scale reactors in a wide range of temperatures with a synthetic wastewater; (ii) operation with a pilot scale reactor (150 L) treating reject water in a municipal wastewater treatment plant; (iii) theoretical study with one-dimensional biofilm model.

5.2 Materials and methods

5.2.1 Experimental set-ups, wastewater and inoculum

There different set-ups (I-III) were used for experimental tests of nitritation, all them were aerobic granular sludge airlift reactors. The main differences in terms of reactor volume, instrumentation and control, wastewater and inoculums are described in Table 5.1. Synthetic wastewater composition was: 4.6 g L^{-1} NH_4Cl (1.2 g of $N\text{-}NH_4^+$ L^{-1}) and the following compounds (in mg L^{-1}): CH_3COONa, 48.0; $CaCl_2 \cdot 2H_2O$, 3.0; KH_2PO_4, 13.0; NaCl, 9.0; $MgCl_2 \cdot 7H_2O$, 6.0; $FeSO_4 \cdot 7H_2O$, 0.13; $MnSO_4 \cdot H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.13; $CuSO_4 \cdot 5H_2O$, 0.07; and H_3BO_3, 0.007. Composition of the real reject wastewater was rather variable with the following concentrations: ammonium: 500-850 mg N L^{-1}, total organic carbon, TOC: 240 – 696 mg C L^{-1}, total inorganic carbon, TIC: 358 – 723 mg C L^{-1}. Variations within these ranges were related to the efficiency of the anaerobic digestion. Dissolved oxygen in the reactors was controlled at different values, in the range (2-7) mg O_2 L^{-1}. Temperature was kept constant at different values, in the range (20 – 30) ºC. The pH was maintained at 7.5 in the reactor bulk liquid through the addition of solid Na_2CO_3.

5.2.2 Analytical methodology

Regular sampling of the bulk liquid of the pilot plant was carried out to determine the total ammonia nitrogen (TAN = $N\text{-}NH_4^+$ + $N\text{-}NH_3$), total nitrite nitrogen (TNN = $N\text{-}NO_2^-$ + $N\text{-}HNO_2$), and nitrate concentrations through off-line analysis. The TAN concentration in off-

line liquid samples withdrawn from the bulk liquid was analyzed by means of a continuous flow analyzer. The TNN and nitrate concentrations were measured by ion chromatography (ICS-2000 Integrated Reagent-Free IC System, DIONEX). Volatile solids (VS), total solids (TS), and sludge volumetric index (SVI) were determined according to APHA standards (APHA, 1995). The granular biomass was characterized throughout the whole experimental periods in terms of size, aspect, shape, granule density, and settling velocity (see Bartroli et al. (2010) for further details).

The fluorescence in situ hybridization (FISH) technique coupled with confocal laser scanning microscopy (CLSM) was used to determine the fractions of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the granules. A detailed description of the applied methodology can be found in Bartrolí et al. (2010). For each determination at least 70 granules were used.

5.2.3 Model development

A one-dimensional biofilm model was developed to simulate the aerobic granular sludge airlift reactor performance based on Wanner and Reichert, (1996) and implemented in the software package AQUASIM, v.2.1d (Reichert, 1998).

The biomass species described as particulate compounds in the biofilm matrix were four: AOB, NOB, heterotrophic bacteria and inert biomass. Total biofilm area was defined as a function of granule size and number of granules. A detachment rate was used to keep a constant biofilm thickness in steady state at a predefined value. Detached biomass from the biofilm was considered as active following the same kinetics defined for the biomass in the biofilm. Attachment of biomass onto the biofilm surface has been neglected. For the sake of simplicity external mass transfer has been neglected. The porosity of the biofilm was fixed as 80% and kept constant during all the simulations. Initial fractions of particulate compounds were 10% AOB, 8% NOB and 2% heterotrophic biomass. Growth of AOB and NOB included inhibition by free ammonia (FA) and free nitrous acid (FNA) as proposed by Jubany et al. (2008). The microbial kinetics and the stoichiometry used as well as other parameters related to the biofilm are detailed elsewhere (Jemaat et al., 2013).

5.2.4 Ratio control strategy

Two different closed loops were implemented: (i) one to maintain the TAN concentration in the bulk liquid (i.e., the reactor effluent, considering a well-mixed liquid phase in the reactor) and (ii) a second one to control the DO concentration in the bulk liquid. To illustrate the control strategy, a conventional block diagram is presented in Fig. 5.1. The variables measured for the two closed loops were the TAN concentration and the DO concentration, whereas the two manipulated variables were the wastewater inflow rate fed to the reactor and the air flow-rate, respectively (see Fig. 5.1 for details). With this control configuration, the setpoints of both closed control loops ($[TAN]_{SP}$ and $[DO]_{SP}$) do not depend on either of the measured variables. Both control loops act independently varying the corresponding manipulated variables (wastewater inflow rate and air flow rate, respectively) on demand to keep the corresponding set points. A proportionality constant has been defined for the ratio station: $R_{SP} = [DO]_{SP}/[TAN]_{SP}$. For further details regarding the ratio control strategy see Bartrolí et al. (2010). These two control loops were also inserted in the model, thus allowing for simulations with DO and TAN under control. For specific equations used in Aquasim implementation see Jemaat et al. (2013).

The maximum value of R_{SP} that could lead to full nitritation in steady state was denoted as $R_{max,fN}$, and it was narrowed within (0.25-0.35) mg O_2 mg^{-1} (Bartroli et al., 2010).

Table 5.1 Description of reactor set-ups, type of wastewater and inoculums used.

Set-up	Reactor volume (L)	Instrumentation and control	Wastewater	Inoculum
I	2.6	DO, T, pH	Synthetic	Aerobic granular sludge previously treating sewage (Jemaat et al., 2012)
II	112	DO, T, pH, NH_4^+	Synthetic	Activated sludge + activated carbon particles (removed after inducement of granules) (Bartroli et al., 2010)
III	150	DO, T, pH, NH_4^+	Reject water	Activated sludge; granulation in SBR and later switching to continuous operation (Torà et al., *under review*)

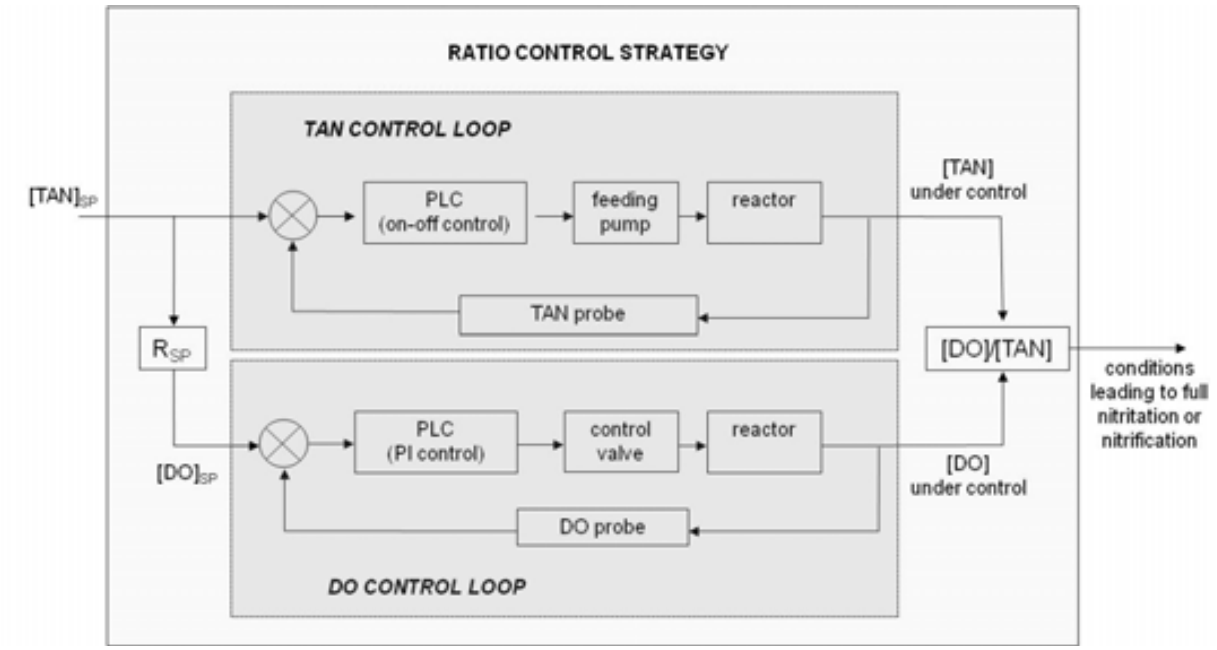

Figure 5.1 Block diagram of the automatic control for partial nitrification to nitrite in biofilm reactors (ANFIBIO) used to ensure the required oxygen-limiting conditions in the biofilm to obtain and maintain continuous full nitritation under stable operating conditions (adapted from Bartrolí et al. (2010)). The ratio station is applied to the set points.

5.3 Results and discussion

5.3.1 Reactor operation

Although different types of start-up were used for each one of the set-ups (see Table 5.1), common features were fast nitrite build up and (total) absence of nitrate in the reactor effluent. For set-up III (real reject water), sequencing batch reactor (SBR) operation was first applied to develop AOB granular sludge, which was possible in few weeks, with nitrate

concentrations in the effluent always lower than 4 mgN L^{-1} after only ca. 10 days of operation. After two months, granular sludge was fully developed (size 0.5 mm; sludge volumetric index ratio at 5 and 30 minutes was measured as SVI_{30}/SVI_5=1.04). SBR operation was then switched to continuous mode maintaining good granular sludge quality (i.e. high settling velocity: 40 m h^{-1} and with SVI_5/SVI_{30}=1) and full nitritation (Torà et al., *under review*).

Operating at DO/TAN concentration ratio lower than 0.25 assured full nitritation for all reactor set-ups with different granule sizes (in the range (0.5-1.1) mm) and operating at different reactor temperatures (20 ºC and 30 ºC).

The highest loading rates achieved required of sludge recirculation events in set-ups II and III. Apparently, the TAN control loop is not able to exceed ca. 1 kgN m^{-3} d^{-1} if sludge is not recirculated, this happened in all three set-ups. In set-up III sludge recirculation events produced immediate increase in loading rate which resulted in stable higher biomass concentration in the reactor after few days without affecting the granular sludge quality (Torà et al., *under review*).

Overall, the ratio control strategy described in Fig. 5.1 produced stable full and partial nitritation. In Table 5.2 and 5.3 we show how ca. 50% oxidation of ammonium to nitrite with absence of nitrate was obtained in set-ups I and III, at relatively high nitrogen loading rate (1 and 4 kg m^{-3} d^{-1}, respectively). Therefore ANFIBIO demonstrated good performance in case of its eventual combination with Anammox for autotrophic N removal.
NOB persisted in the granules, although in very small numbers (<1%), despite months of operation with hardly any nitrate in the effluent.

Table 5.2 Summary of results obtained when applying a ratio control strategy for nitritation in aerobic granular sludge airlift reactors. R_{SP} is the proportionality constant of the ratio control strategy; n.c. = not computed; * recirculation of sludge using a small external settler (i.e. 5 and 20 L for set-ups II and III respectively). Complementary information for each period is found in Table 5.3.

Set-up	Period	Temperature (ºC)	R_{SP} (mgO_2 mg^{-1}TAN)	TAN converted to TNN (%)		TAN converted to nitrate (%)		Flow-rate (L d^{-1})	
				exp.	model	exp.	model	exp.	model
I	A	30	0.04	96	n.c.	1	n.c.	1.33	n.c.
I	B	30	0.004	52	n.c.	1	n.c.	2.9	n.c.
II	A	30	0.17	96	98	1	0	61	93
II	B	30	0.35	1	4	98	94	93	80
II	C	30	0.25	98	98	1	0	79	74
II	D	30	0.18	98	97	1	0	93	117
II	E	30	0.18	98	97	1	0	570*	516
II	F	20	0.15	98	98	1	0	56	60
III	A	30	0.14	98	n.c.	1	n.c.	200	n.c.
III	B	30	0.10	98	n.c.	1	n.c.	345*	n.c.
III	C	30	0.02	50	n.c.	1	n.c.	805*	n.c.

Table 5.3 Complementary characterization and reactor conditions for each steady state described in Table 5.2. Note how measured DO and TAN concentration in the reactor effluent are ca. equal to setpoints. The pH was maintained at 8.2 in all periods. n.m. = not measured.

Set-up (period)	Loading rate (kgN m^{-3} d^{-1})	$[DO]_{SP}$ (mgO_2 L^{-1})	$[TAN]_{SP}$ (mgN L^{-1})	Size (mm)	Reactor biomass concentration (gVS L^{-1})	Solids concentration in effluent (gVS L^{-1})	Granule density (gVS $L_{particle}^{-1}$)
I(A)	0.4	2	50	0.8	3.3	0.03	200
I(B)	1.0	2	460	1.1	3.4	0.05	110
II(A)	0.8	5	30	0.9	0.6	<0.1	38
II(B)	1.0	7	20	0.7	0.6	<0.1	73
II(C)	0.9	5	20	0.9	0.6	<0.1	38
II(D)	1.0	7	40	0.7	0.6	<0.1	64
II(E)	6.1	7	40	0.7	4.6	<0.1	67
II(F)	0.6	4.5	30	0.7	0.8	<0.1	40
III(A)	0.9	5.5	40	0.5	1.6	0.2	98
III(B)	1.7	4	40	0.4	5.5	0.2	n.m.
III(C)	4.0	5.5	305	0.4	5.0	0.2	n.m

5.3.2 Influence of the influent TAN concentration

To investigate the effect of influent TAN concentration, we took experimental conditions in steady state IIC (see Table 5.2 and 5.3) and run different simulations to determine the applied NLR_v, flow-rate and bulk biomass concentration of AOB for each value of $[TAN]_{influent}$ tested (in the range 400-1200 mg N L^{-1}). Setpoints were fixed at $[TAN]_{SP}$=20 mg N L^{-1} and $[DO]_{SP}$ = 5 mg O_2 L^{-1}. In addition, $R_{max,fN}$ for each case was estimated maintaining a fixed value of $[TAN]_{SP}$ =20 mg N L^{-1} and manipulating $[DO]_{SP}$ (see Fig. 5.2). Results presented in Fig. 5.2 show how influent TAN concentration has a clear influence on reactor performance, both in terms of NLR_v and with regard to the value of $R_{max,fN}$. Lower influent TAN concentrations led to lower NLR_v in steady state, see Fig. 5.2. In addition, lower influent TAN concentrations led to lower $R_{max,fN}$, i.e. the achievement of full nitritation required a lower $[DO]_{SP}$ (see Fig. 5.2).

When analyzing the results we found different biomass concentration in suspension (coming from detachment) for each influent TAN concentration tested (see Table 5.4). This turned to be the main reason behind this behavior. When the influent TAN concentration decreased, the flow-rate increased, and therefore a lower concentration of biomass in suspension was obtained (i.e., the rate of washed out biomass in suspension increased). Overall, this means that a lower total biomass concentration is retained in the reactor, and therefore a lower NLR_v is achieved in steady state. This is how the inflow rate is playing a role and has a defined

influence in the achievement of full nitritation in biofilm reactors. This explanation was supported by the following simulation: at an influent TAN concentration of $[TAN]_{influent}$ =600 mg N L^{-1}, 34.8 mg COD L^{-1} of AOB were continuously supplied to the bulk liquid (as biomass in suspension) in the simulation, as to equal $[X]_{AOB}$=77.7 mg COD L^{-1}, which was the value achieved at $[TAN]_{influent}$ =1200 mg N L^{-1} (see Table 5.4). The NLR_v obtained was ca. 1 g N L^{-1} d^{-1}, equivalent to that obtained at $[TAN]_{influent}$ =1200 mg N L^{-1} (see Table 5.4).

The model predicted a strong dependence between the suspended biomass concentration of AOB and the steady loading rate achieved (see Table 5.4). This could be the reason why the recirculation events performed in set-ups II and III (see previous discussion in the section *Reactor operation*), enhanced the fast increase in nitrogen loading rate, which subsequently resulted in a (stable) increase of the granular sludge concentration, i.e. promoting new granule development, in the periods IIE and IIIB (Tables 5.2 and 5.3).

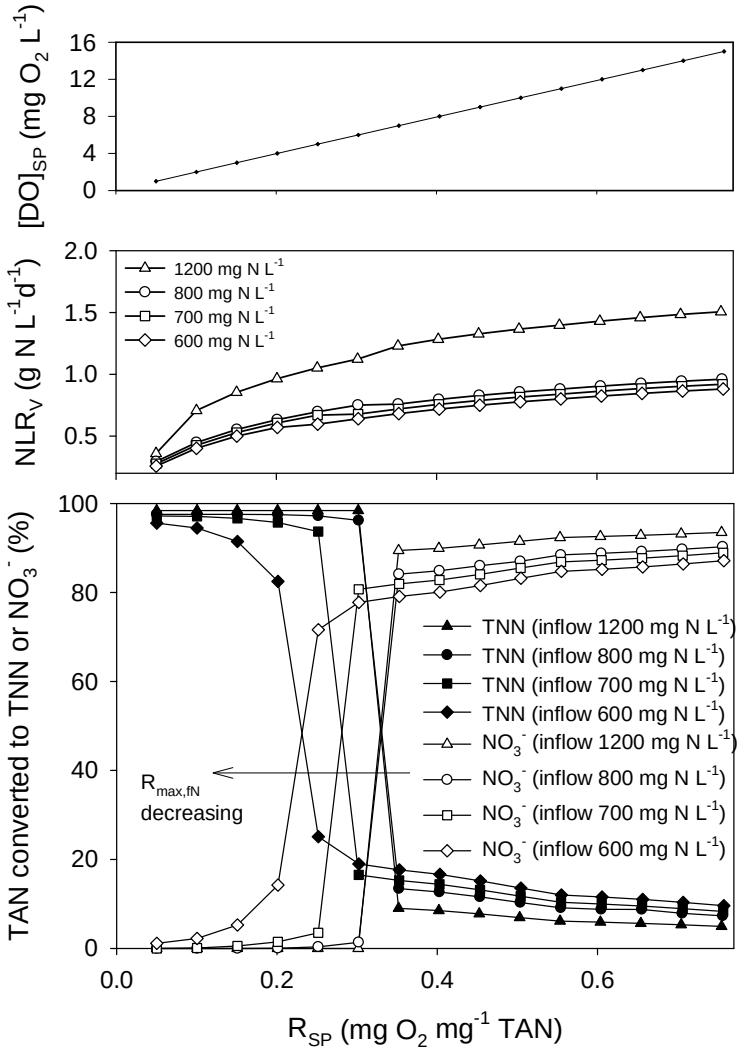

Figure 5.2 Influence of the influent TAN concentration on conversion at different setpoints ratios (R_{SP}). All simulated values were computed keeping a fixed total ammonia nitrogen concentration setpoint $[TAN]_{SP}$ = 20 mg N L^{-1} and manipulating dissolved oxygen concentration setpoint ($[DO]_{SP}$). Temperature 30 ºC, granule size of 0.9 mm. The arrow indicates that the maximum value of R_{SP} leading to full nitritation ($R_{max,fN}$) was decreasing as the inflow TAN concentrations decrease. The applied volumetric nitrogen loading rate (NLR_v) for each $[DO]_{SP}$ tested was also plotted.

Table 5.4 Simulation study to determine the effect of different influent TAN concentrations ($[TAN]_{influent}$) on full nitritation. Setpoints were fixed at $[TAN]_{SP}$=20 mg N L^{-1} and $[DO]_{SP}$ = 5 mg O_2 L^{-1} (i.e., constant R_{SP} = 0.25). For each value of $[TAN]_{influent}$ the applied NLR_v, the flow-rate and the suspended biomass concentration of AOB ($[X]_{AOB}$) were found. In addition, $R_{max,fN}$ for each case was estimated keeping a fixed value of $[TAN]_{SP}$=20 mg N L^{-1} and manipulating $[DO]_{SP}$ (see Fig. 5.2).

$[TAN]_{influent}$ (mg N L^{-1})	$[X]_{AOB}$ (mg COD L^{-1})	Flow-rate (L d^{-1})	NLR_v (g N L^{-1} d^{-1})	$R_{max,fN}$ (mgO_2 mg^{-1}TAN)
1200	77.7	73.6	1.05	0.30
800	53.4	97.8	0.70	0.25
700	47.2	107.1	0.67	0.15
600	42.9	111.4	0.60	0.05

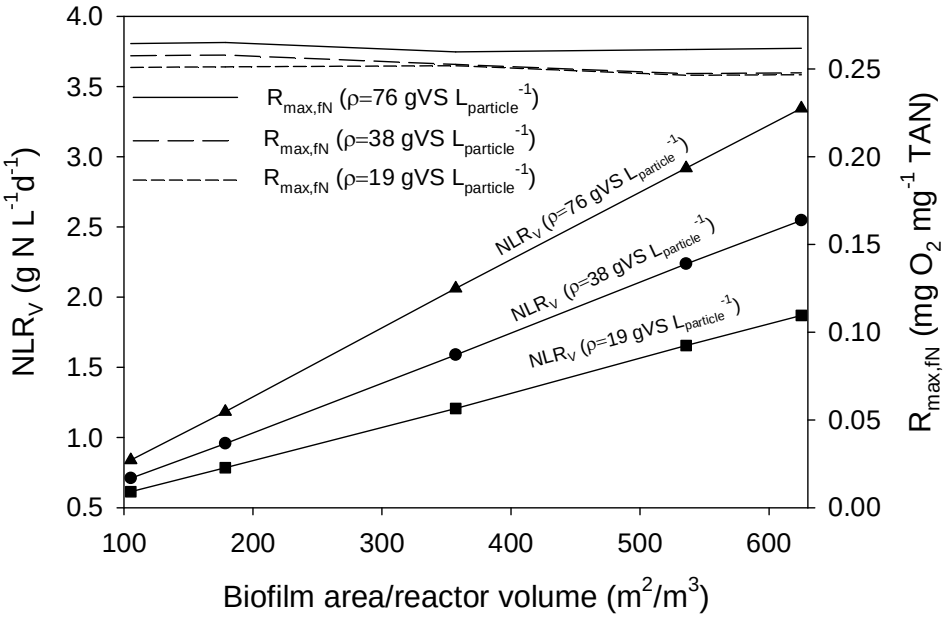

Figure 5.3 Effect of different biofilm density on the maximum value of setpoints ratio leading to full nitritation ($R_{max,fN}$) and on the applied volumetric nitrogen loading rate (NLR_v). Different biofilm areas were tested and expressed per unit of reactor volume. For all simulations, temperature was 30 ºC, granule size of 0.9 mm and the dissolved oxygen concentration setpoint was kept constant at $[DO]_{SP}$= 5 mg O_2 L^{-1}.

5.3.3 Influence of biofim density on the achievement of full nitritation

To investigate the influence of the biofilm density on $R_{max,fN}$, the following procedure was followed: experimental conditions for steady state IIC (see Table 5.2 and 5.3) were used with the exception of the number of granules. The number of granules was manipulated to modify

the total biofilm area (without varying the granule size) for a wide range of values of the biofilm area to reactor volume ratio. To determine the $R_{max,fN}$ value in each case, the $[DO]_{SP}$ was kept constant at 5 mg O_2 L^{-1}, whereas $[TAN]_{SP}$ was manipulated to find the lowest $[TAN]_{SP}$ value at which full nitritation is achieved (i.e., $R_{max,fN}$). The results of this iterative procedure were repeated for a set of different biofilm densities (14, 38, 76 g VS $L_{particule}^{-1}$). The results showed how the values of $R_{max,fN}$ were not affected by the density of the biofilm, producing only minor differences (Fig. 5.3). The obtained NLR_v values were slightly higher for denser biofilms, as expected. Obviously, an increase of the biofilm area (by increasing the number of granules in the reactor) led to a higher NLR_V (see Fig. 5.3).

5.4 Conclusions

Suitable stable effluent for Anammox has been obtained using the desired TAN setpoint (i.e. 50% of influent ammonium oxidation). Recirculation events enhance the achievement of high rate nitritation due to recirculation events. High influent ammonium concentration enhanced full nitritation, as predicted by the model. Poor influence of the biofilm density in the achievement of full nitritation was found with the simulation study.

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PART III

SIMULTANEOUS NITRITATION AND PHENOLIC COMPOUNDS REMOVAL

Part of this chapter was published as:

Jemaat, Z., Fernández, I., Suárez-Ojeda, M.E., Pérez, J., Carrera, J., 2012. Could be feasible an autotrophic BNR process for treating complex industrial wastewaters containing ammonium and phenolic compounds? In: Proceedings of the IWA Nutrient Removal and Recovery 2012: Trends in NRR, Harbin, China: International Water Association, 116-117.

Jemaat, Z., Carrera, J., Suárez-Ojeda, M.E., Pérez, J., 2013. Simultaneous *o*-cresol removal and partial nitritation using aerobic granular sludge operating in continuous mode. 9th International Conference on Biofilm Reactors, Paris, France; Internationl Water Association, May 2013.

Part of this chapter is being prepared for publishing as:

Jemaat, Z., Suárez-Ojeda, M.E., Carrera, J., Pérez, J., Partial nitritation and *o*-cresol removal in a continuous airlift reactor using granular biomass. (*In preparation*)

Jemaat, Z., Suárez-Ojeda, M.E., Carrera, J., Pérez, J., Simultaneous nitritation and p-nitrophenol removal in a continuous airlift reactor using granular sludge. (*In preparation*)

Jemaat, Z., Suárez-Ojeda, M.E., Carrera, J., Pérez, J., Effects of sequentially alternating pollutant (SAP) feeding on the aerobic granular reactor performing simultaneous partial nitritation and *o*-cresol biodegradation. (*In preparation*)

Jemaat, Z., Suárez-Ojeda, M.E., Carrera, J., Pérez, J., Impact of starvation and subsequent reactivation on the aerobic granular reactor treating simultaneously ammonium and phenolic compound. (*In preparation*)

Chapter 6

Partial nitritation and *o*-cresol removal in a continuous airlift reactor using granular sludge

Summary

Several chemical industries produce wastewaters containing both, ammonium and phenolic compounds. The biological treatment of these wastewaters could be regarded as the preferred choice compared to the physicochemical ones, due to its advantages in terms of environmental footprint and operational costs reduction. In this study, the simultaneous partial nitritation and *o*-cresol biodegradation using granular sludge in a continuous airlift reactor is proposed as an alternative to treat those industrial wastewaters. A nitrifying granular sludge developed in a continuous airlift reactor for treating a high-strength wastewater containing 950 ± 25 mg $N\text{-}NH_4^+$ L^{-1} was utilised in this study. On day-0, the airlift reactor was bioaugmented with a *p*-nitrophenol-degrading activated sludge and *o*-cresol was added progressively to the feed up to achieving 100 mg L^{-1}. The results showed that stable partial nitritation process and full biodegradation of *o*-cresol were simultaneously maintained for more than 100 days of operation. The achieved nitrogen loading rate (NLR_v) and *o*-cresol loading rate (CLR_v) were 1.1 g N $L^{-1}d^{-1}$ and 0.11 g *o*-cresol $L^{-1}d^{-1}$, respectively. Two *o*-cresol shock-load events with concentrations of 300 mg L^{-1} and 1000 mg L^{-1} were applied. In spite of these shock load events, the partial nitritation process was kept stable and *o*-cresol was totally biodegraded, achieving among the highest CLR_v ever reported. The results from the fluorescence in-situ hybridization (FISH) technique showed that heterotrophs bacteria able to degrade *o*-cresol tends to locate at the outer layer, and ammonia oxidising bacteria (AOB) tends to locate in the inner layers of the granule.

6.1 Introduction

Several industrial processes such as petroleum refinement, coking operations, coal processing, petrochemicals manufacturing, plastics paints and resins production release wastewaters containing both, ammonium and phenolic compounds (Morita et al., 2007; Milia et al., 2012). Often, *o*-cresol is being one of the commonly found phenolic compounds in those types of industrial wastewaters (Gallego et al., 2008). A biological treatment by means of nitritation-denitrification is nowadays regarded as one of the lowest footprint technologies for removing nitrogen from ammonium-rich wastewaters (Ahn, 2006). Nevertheless, the presence of phenolic compounds in industrial wastewaters could advise for (expensive) physicochemical treatments due to the potential inhibitory or toxic effects over a biological treatment (Oller et al., 2011). In particular, the inhibition of nitrifying microorganisms by phenolic compounds is well documented (Amor et al., 2005; Liu et al., 2005; Morita et al., 2007). Specific studies on the *o*-cresol inhibition over nitrifying were also reported (Tomlinson et al., 1966; Dyreborg and Arvin, 1995). In spite of these challenges, the development of a biological treatment dealing simultaneously with phenols and ammonium rich wastewaters would be desirable, among others, because of the low costs associated to this type of treatment. .

Among the wide spectrum of reactor systems used in wastewater treatment, those based on granular sludge could become a robust alternative for the treatment of wastewaters containing inhibitory or toxic organic compounds (Maszenan et al., 2011). The diffusion gradients existing in aerobic granules could contribute to reduce the inhibitory effect of these compounds because the granule architecture could act as a protective shield to sensitive bacteria (Liu et al., 2005; Morita et al., 2007; Maszenan et al., 2011). Development of granular sludge is commonly achieved via sequencing batch reactor (SBR), by applying short settling times and high shear stress (Gao et al., 2011). However, conventional batch operation is not advisable for the treatment of recalcitrant compounds, and alternative strategies like distributed feeding along the cycle have been proposed to minimize substrate inhibition (Martín-Hernández et al., 2009). Continuous mode of operation would circumvent this drawback since the reactor concentration of the recalcitrant compound is expected to be low due to the high removal efficiency, therefore mitigating the toxic effects in the reactor.

In the present study, a continuous granular sludge reactor performing nitritation of high-strength ammonium wastewater was bioaugmented with activated sludge specialized in the degradation of phenolic compounds with the main aim to develop a granular sludge with a special architecture, in which an external layer of heterotrophs is degrading the phenolic compound and, at the same time, this external shell is protecting ammonia oxidising bacteria (AOB) against this potentially inhibitory/toxic compound. The results will serve to assess the feasibility of a biological treatment for simultaneous partial nitritation (potentially to feed an Anammox reactor for nitrogen removal) and *o*-cresol removal.

6.2 Materials and Methods

6.2.1 Experimental set-up

A glass airlift reactor with a working volume of 2.6 L was utilised 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. Figure 6.1 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). 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). 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.

6.2.2 Reactor conditions and inoculums

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 continuously fed using a synthetic wastewater aiming to obtain partial nitrification. Before bioaugmentation of *p*-nitrophenol (PNP) degrading activated sludge was carried out, the performance of the reactor on day-0 was with average effluent concentrations of: total ammonia nitrogen (TAN), 323 ± 92 mg N L^{-1}; total nitrite nitrogen (TNN), 628 ± 45 mg N L^{-1}); nitrate, 29 ± 15 mg N L^{-1} and volumetric nitrogen loading rate (NLR_v) of 0.4 ± 0.1 g N $L^{-1}d^{-1}$. The operating conditions of the reactor in the present study and the main characteristics of the granular biomass at day-0 are detailed in Table 6.1. Additionally, the pH of the reactor was maintained by a regular addition of $NaHCO_3$ into the reactor.

6.2.3 Wastewater composition

The airlift reactor was fed with synthetic wastewater with 3.63 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.

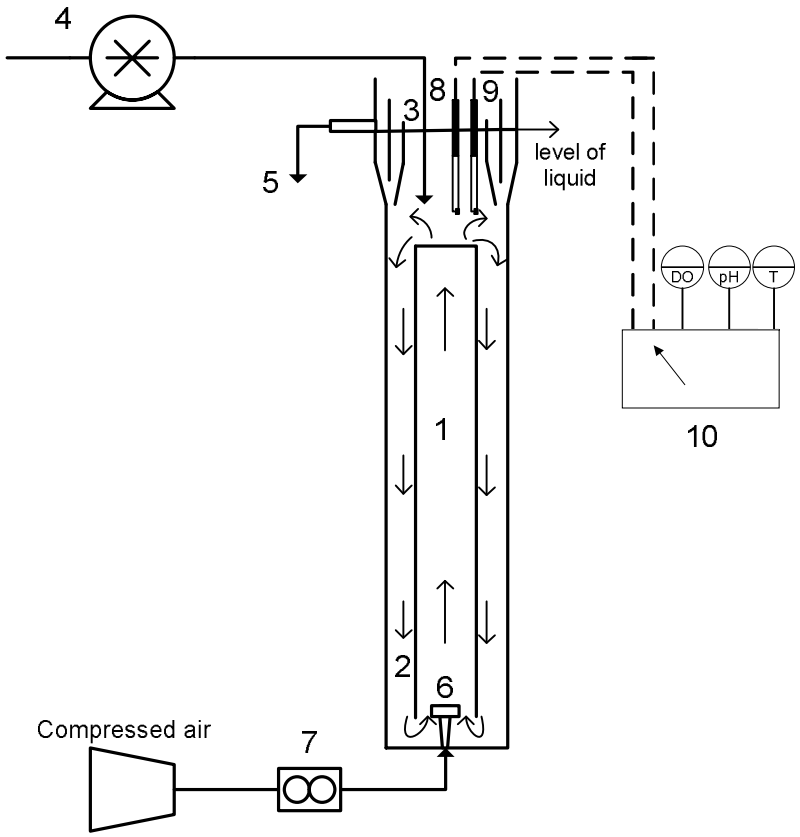


Figure 6.1 Experimental set-up of the continuous granular airlift reactor. (1) riser; (2) down comer; (3) separator; (4) feed pump; (5) effluent port; (6) air sparger; (7) rotameter; (8) pH probe; (9) DO probe; (10) monitoring panel.

Table 6.1 The operating conditions of the reactor during the study period and the main characteristics of the granular biomass at day-0.

Reactor conditions	
Temperature (ºC)	30 ± 1
Dissolved oxygen (mg O_2 L^{-1})	2.0 ± 0.3
pH	8.3 ± 0.2
Hydraulic retention time (HRT) (d)	0.85 to 2.3
Air flow rate (mL min^{-1})	250 ± 50
Biomass characteristics	
Mean size (mm)	1.5 ± 1.2
Biomass density (g VSS $L_{particle}^{-1}$)	290 ± 120
Settling velocity (m h^{-1})	61 ± 24
Sludge volumetric index (SVI_5) (mL g^{-1} TSS)	8.1
Ratio SVI_5/SVI_{30}	1.0

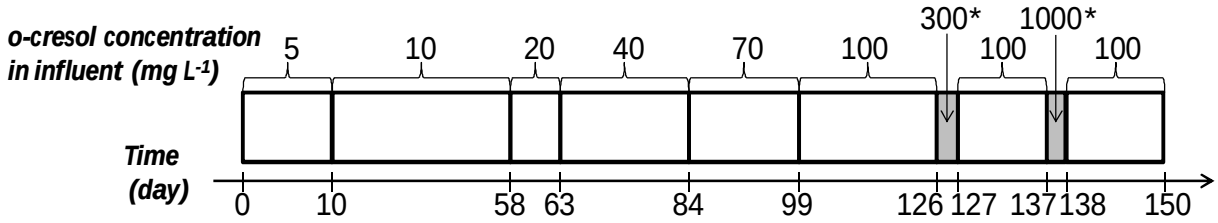
6.2.4 Bioaugmentation, the operational strategy and shock load events

On day-0, the airlift reactor was bioaugmented with 500 mL (2 g L^{-1} VSS) of activated sludge from a sequencing batch reactor performing stable PNP degradation (Martín-Hernández et al., 2009). The bioaugmented biomass was accounted around 12 % of the total volatile suspended solids (VSS) inside the reactor. This percentage was selected based on the suggestion of Martín-Hernández et al. (2012) to use minimum 5 % w/w of bioaugmented biomass for ensuring the biomass is retained in the reactor for a long period. Considering that our reactor was operating in continuous mode and biomass wash out could be obvious, an excess of bioaugmented biomass was added.

On day-0, *o*-cresol was added to the synthetic wastewater and this wastewater was fed into the reactor. *O*-Cresol concentrations in the influent were progressively increased during the whole study period (Figure 6.2).

On day 126 and 137, perturbation on the *o*-cresol loading rate were performed on the reactor with the addition of *o*-cresol at concentrations of 300 and 1000 mg L^{-1}, respectively. Each shock load event was applied for 24 h and then, the operation was switched back to the normal feeding influent (950 mg N-NH_4^+ L^{-1} and 100 mg *o*-cresol L^{-1}) as detailed in Figure 6.2.

Figure 6.2. *O*-Cresol feeding strategy imposed during the continuous operation of the granular airlift reactor. Influent containing also ammonium concentration of 950 ± 25 mg N-NH_4^+ L^{-1}.



*shock load experiment for a period of 24 h.

6.2.5 Analytical methods

O-Cresol was determined by High Performance Liquid Chromatography (HPLC) as detailed elsewhere (Martín-Hernández et al., 2009). The ammonium concentration measured as total ammonia nitrogen (TAN), the nitrite as total nitrite nitrogen (TNN) and nitrate concentrations were measured as detailed elsewhere (Bartrolí et al., 2010; Isanta et al., 2012). 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 term of size, granule density and settling velocity. 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. Density of the granular biomass was determined using the Dextran Blue method described by Beun et al., (2002). 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). Moreover, the extracellular polymeric substances (EPS) were extracted from the granules using formaldehyde + NaOH and were analyzed according to Adav and Lee, (2011).

The main chemicals used in this study, *o*-cresol (concentrated solution, purity 99%) and ammonium chloride (purity 99.5%) was 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.

6.2.6 FISH analysis

The fluorescence in-situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM) was used to identify possible heterotrophic bacteria able to degrade *o*-cresol, betaproteobacteria ammonia-oxidizing bacteria (β-AOB) and nitrite-oxidizing bacteria (NOB) along the geometry of 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. Hybridizations were carried out using probes targeting specific microorganisms as described in Table 6.2. The general probe consisting of equal parts of UNIV1390 and EUBmix was used for detection of all bacteria. The probes for identify possible heterotrophic bacteria able to degrade *o*-cresol were selected taking into account the characterisation of the PNP-degrading biomass bioaugmented to our reactor previously done by Suárez-Ojeda et al. (2011). FISH protocol was also adapted from the same authors. A Leica TCS-SP5 AOBS confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with objective HC PL APO CS 63x1.25 oil equipped with two He-Ne lasers and hybrid detector was used.

Table 6.2 Probes targeting specific microorganisms employed in the FISH analysis

Probe name	**Specificity**	**Reference**
Nso190	β-AOB	Mobarry et al. (1996)
NIT3	*Nitrobacter* sp.	Wagner et al. (1996)
KO 02	*Arthrobacter* sp.	Franke-Whittle et al. (2005)
ACA652	Genus *Acinetobacter*	Wagner et al. (1994)
UNIV1390	All organisms	Zheng et al. (1996)
EUBmix	Most bacteria, planctomycetales and verrucomicrobiales	Daims et al. (1999)

6.3 Results and discussion

6.3.1 Performance of a granular airlift reactor for simultaneous removal of ammonium and *o*-cresol

The performance of partial nitritation and *o*-cresol biodegradation in a granular nitrifying reactor treating a high strength ammonium wastewater containing *o*-cresol is shown in Figures 6.3(A)-(C). The reactor was able to maintain stable partial nitritation at exceptionally low nitrate concentrations in the effluent (Figure 6.3A) and with full *o*-cresol biodegradation (Figure 6.3C) for a long-term operation (150 days). On day 35 onward, the effluent [TNN]/[TAN]concentration ratio was steadily maintained between 1.0 and 1.5 during more than 100 days producing a suitable Anammox effluent. The reactor achieved a relatively high volumetric nitrogen loading rate (NLR_v =1.1 ± 0.1 g N $L^{-1}d^{-1}$) in spite of the presence of *o*-cresol in the wastewater. This value of NLR_v is comparable to those reported in the literature for conventional high-strength ammonium wastewater (i.e. 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}). In the whole experimental period, *o*-cresol was completely degrade and achieved a volumetric *o*-cresol loading rate (CLR_v) of 0.11 g o-cresol L^{-1} d^{-1}. The CLR_v value achieved in the present study is close to those reported for *o*-cresol biodegradation in a continuous reactor, Perron and Welander, (2004) (0.1 g *o*-cresol L^{-1} d^{-1}) and Gallego et al. (2008) (0.192 g cresols L^{-1} d^{-1}). The biomass concentration in the reactor (VSS_R) was stable and maintained at around 3 and 3.5 g L^{-1} during the whole of the operating period (exceptional after shock load events) (Figure 6.6A). In the first ten days of the experimental period, an increased of biomass concentration in the effluent (VSS_E) was observed at around 240 mg L^{-1} (Fig. 6.6A). This occurrence was strongly related to the washout of a fraction of the bioaugmented PNP-degrading activated sludge that could not be retained in the reactor. On day 15 onward, the VSS_E remained stable between 40 and 90 mg L^{-1} in most of the experimental period showing the stability of granular sludge.

Our results showed that nitrite oxidation was always prevented due to the strong oxygen limiting conditions imposed in the reactor. Although the DO concentration in the reactor was not too low (2 mg O_2 L^{-1}), the ammonium concentration was always kept in great excess. Therefore, the [DO] / [TAN] ratio in the reactor was very low (0.003 and 0.015 mg O_2 mg^{-1} TAN), outcompeting NOB in the granular sludge, as already demonstrated in previous studies (Bartrolí et al., 2010; Jemaat et al., 2013).

Bioaugmentation together with the operational strategy selected, by which *o*-cresol was progressively increased in the influent, prevented the accumulation of *o*-cresol in the reactor bulk liquid, as supported by the HPLC analyses showing concentrations of *o*-cresol lower than the detection limit (0.1 mg L^{-1}) throughout the whole experimental period (with the exception of the shock load events) and without detection of any potential intermediates. This was crucial for the stability of the nitritation process since the inhibition of nitrifiers by phenolic compounds especially AOB is well documented (Amor et al., 2005; Morita et al., 2007; Milia et al., 2012)

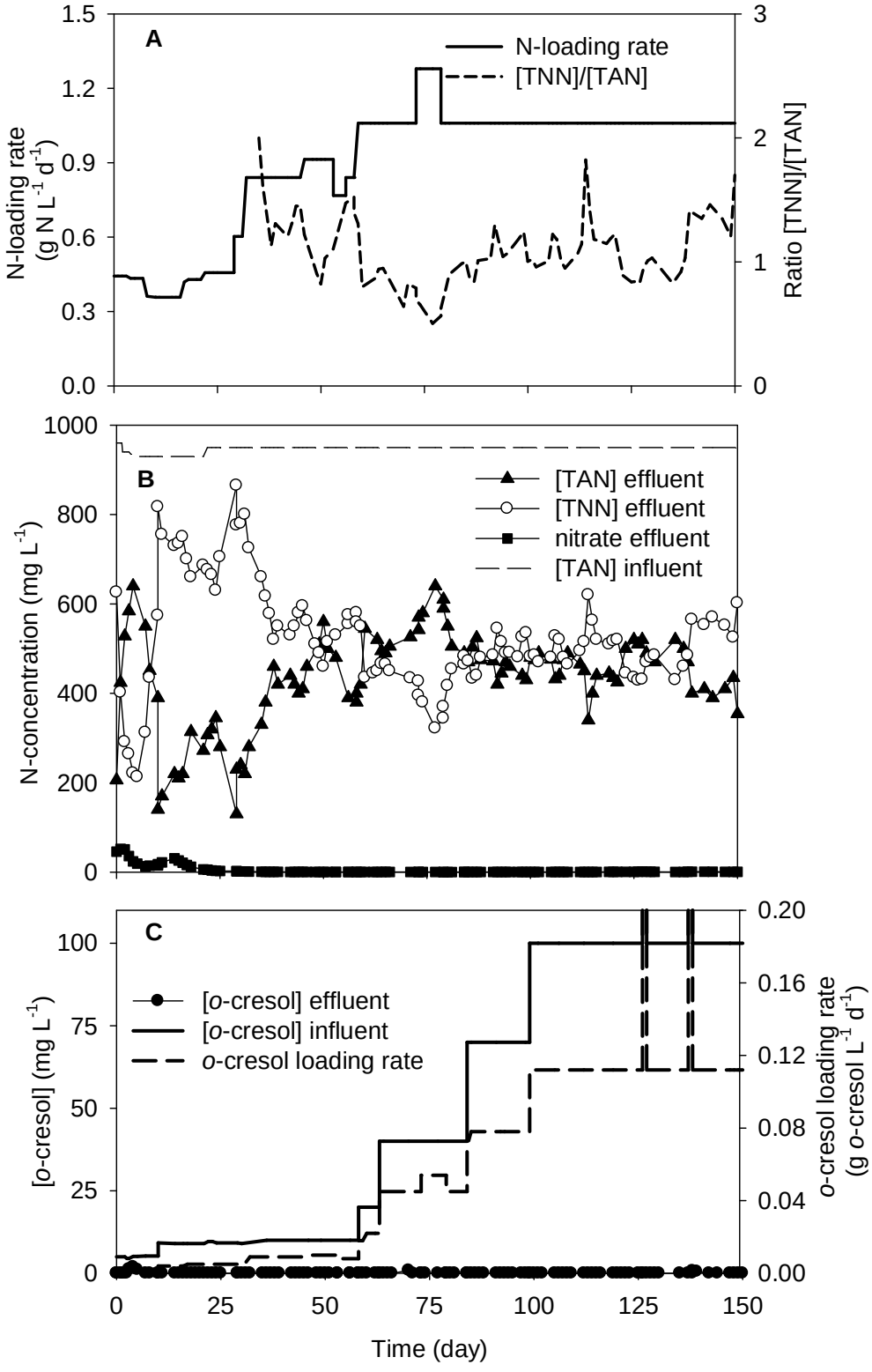


Figure 6.3 Performance of a granular nitrifying reactor treating a high-strength ammonium wastewater containing *o*-cresol. (A) Volumetric nitrogen loading rate (NLR_v) and nitrite/ammonium ratio; (B) Partial nitritation performance; (C) *o*-cresol biodegradation performance during the experimental period. Bioaugmentation was performed on day 0.

A key feature for maintaining simultaneous removal of ammonium and *o*-cresol at long-term is the retention, development and attachment of the specialised biomass over the nitrifying granules. To observe the morphological changes in the nitrifying granules through the experimental period, a closer look to the granules was performed on days 1, 90 and 123 (Figure 6.4). On the first day, the original nitrifying granules were characterised by a smooth and regular shape (Fig. 6.4A). After 90 and 123 days, it was clearly seen that the outer surface of granules were surrounded by filamentous-like heterotrophic bacteria that could be linked to *o*-cresol biodegradation (Fig. 6.4B and 6.4C). On day-123, the filamentous heterotrophic bacteria were even more apparent. This extreme is later confirmed by FISH analysis over sliced granules (see section 6.3.4).

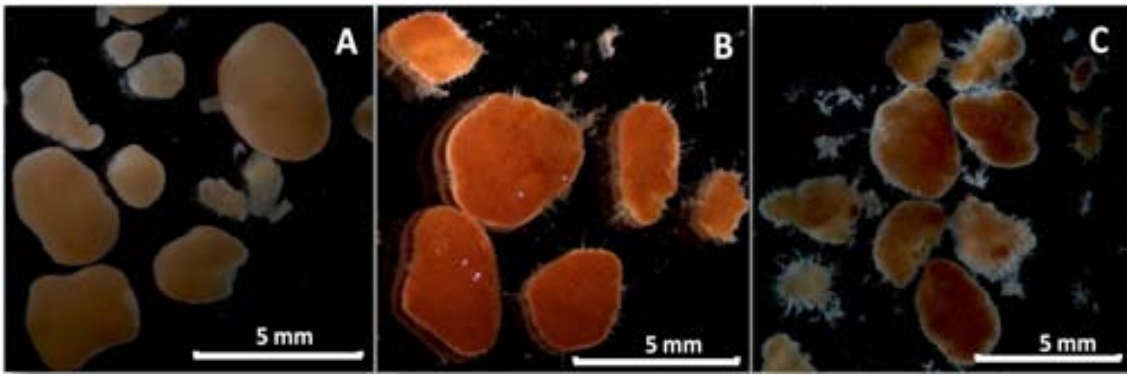


Figure 6.4 The changed of aerobic granular sludge morphology during the simultaneous partial nitritation and *o*-cresol biodegradation. (A) day 1; (B) day 90; (C) day 123. Scale bar = 5 mm.

6.3.2 *o*-Cresol shock load events

On the view of these results, we explored the performance of the reactor in front of *o*-cresol shock load events. The performance of the reactor when two different *o*-cresol shock load events were applied is presented in Figures 6.5A and 6.5B. In these figures, the theoretical accumulation of *o*-cresol concentration in the reactor considering no biological degradation is also depicted to ease the assessment of the impact of the shock load events. In the first shock load event on day 126, *o*-cresol accumulated in the reactor bulk liquid up to 2 mg L^{-1} the first two hours but later, *o*-cresol was fully degraded (Figure 6.5A). Meanwhile, partial nitritation was maintained unaffected. On day 137, the second shock load event was performed, and in the first four hours, *o*-cresol accumulated in the reactor bulk liquid up to 20 mg L^{-1}. Similarly, to the previous shock load event, *o*-cresol was fully degraded (Figure 6.5B) after few hours. During this event, partial nitritation was stably maintained and surprisingly after the shock load event, the nitritation rate was slightly increased at around 14 % and maintained until the end of the experimental period. It is also essential to highlight that in both shock loading events, besides a small *o*-cresol accumulation of few hours, high removal efficiency of *o*-cresol still took place in the reactor. The reactor achieved a CLR_v of 1.1 g *o*-cresol L^{-1} d^{-1} (day 137, shock load of 1000 mg *o*-cresol L^{-1}), and these results seem to indicate that the airlift reactor could be operated to a CLR_v 10 times higher (1.1 g *o*-cresol L^{-1} d^{-1}) than the achieved in normal operation (0.11 g *o*-cresol L^{-1} d^{-1}).

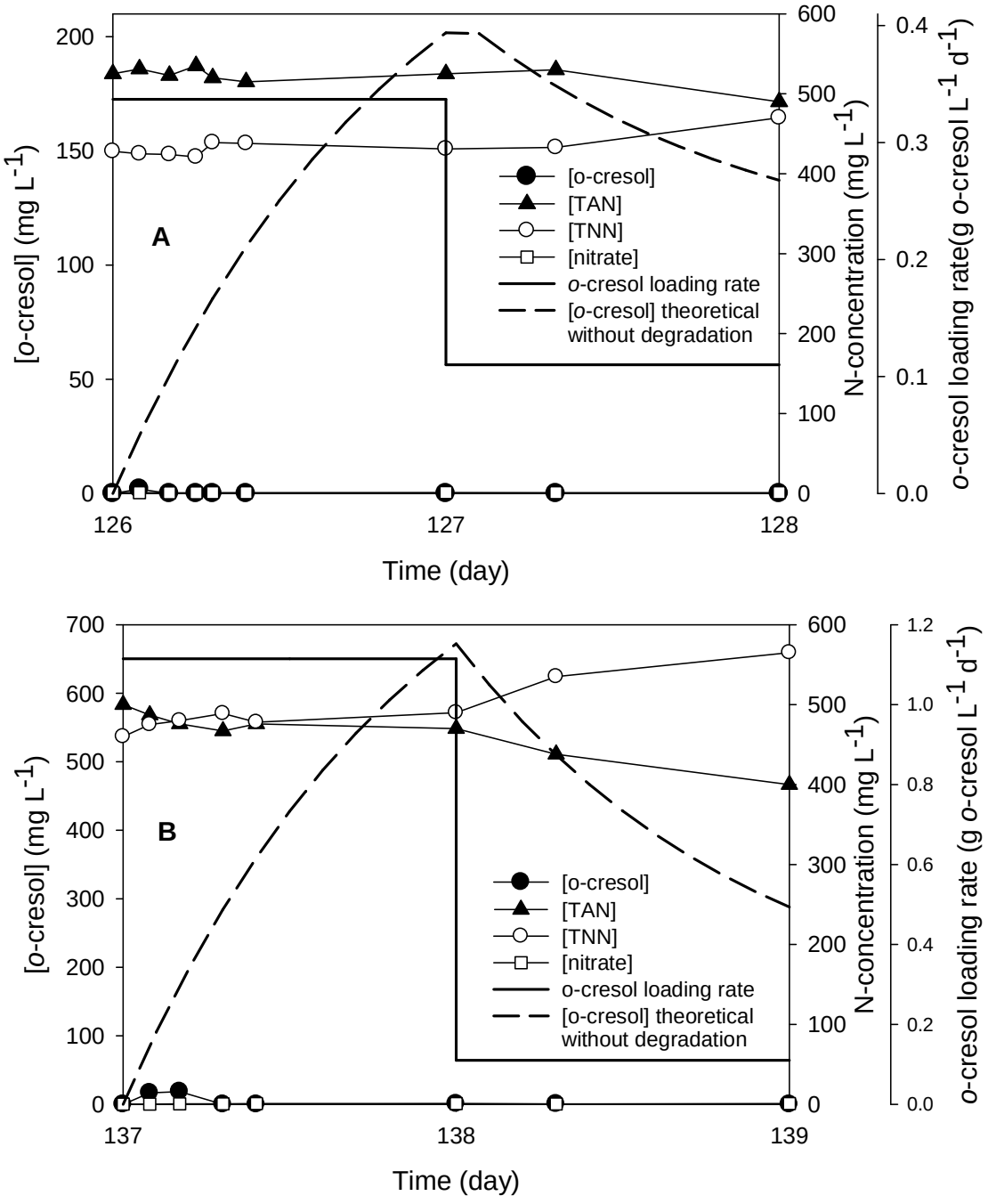


Figure 6.5 Effect of shock loading events on the performance of partial nitritation and *o*-cresol removal. The theoretical accumulation of *o*-cresol concentration in the reactor considering no biological degradation is also depicted for guiding purpose. (A) day-126; (B) day-137.

If this is the case, the CLR_v achieved in the present study is considerably high comparing with the values found in the literatures (Nakhla and Al-Harazin, 1993; Perron and Welander, 2004; Gallego et al., 2008). More long-term experiments are needed to confirm this possibility. Nevertheless, it is also vital to stress that, our reactor was also simultaneously performing partial nitritation, which is the first ever reported using a continuous granular sludge reactor.

After the shock load event on day 137, the VSS_R was decreased to about 2.8 g L^{-1} (Figure 6.6A). The decreased of VSS_R was strongly linked to the noticeable biomass washout between day-127 and 137. A few days after the 300 mg o-cresol L^{-1} shock load event, the VSS_E was increased to 150 mg L^{-1} compared to only 50 mg L^{-1} prior to the shock load event (Figure 6.5A). A possible explanation for this might be that an increased of heterotrophs growth because of the amount of organic substrate available during the shock loading event. As can be seen in Figure 6.4C and 6.6D, heterotrophs growth were dominated on the outer surface of granule and characterized by a filamentous-type, fluffy and loose-structured morphology that are potentially easy to detach from the granule surface. Thus, it is expected that more biomass washout after shock load events, through detachment of biomass from granules as observed in our experiment (Figure 6.5A). Although VSS_R was decreased, the reactor performance was maintained stable. Nevertheless, the VSS_R recovered its previous average value (prior to shock load events) few days after the normal feeding was switched back.

Studies on the *o*-cresol inhibitory impact over nitrification have been reported by Tomlinson et al. (1966) and Dyreborg and Arvin, (1995) who has estimated a value of 12.5 and 1.3 mg *o*-cresol L^{-1} resulted to 75 and 100 % inhibition, respectively. In the present study, we did not observe any nitritation inhibition by *o*-cresol even up to 20 mg *o*-cresol L^{-1} during shock load events (Figure 6.5B). The *o*-cresol non-inhibitory impact over AOB observed in the present study is possibly because of the potential shielding effect of heterotrophs due to a special architecture layer developed over the granule surface. Another possible reason could be that granular sludge had been acclimated to *o*-cresol for a long period before the shock load event took place. This, consequently, should significantly decrease the possible inhibitory effect of *o*-cresol over AOB. Our findings suggest that the formation of a diversified microbial consortium over the granules through a bioaugmentation strategy might help to the faster development of aerobic granules that have a better ability to withstand to shock loads of toxic compounds than suspended nitrifying bacteria. The present findings are consistent with those studies demonstrating that nitrifying bacteria embedded in microbial granules were effective protected from the inhibitory effect of phenolic compounds present in the wastewater (Liu et al., 2005; Jiang et al., 2010).

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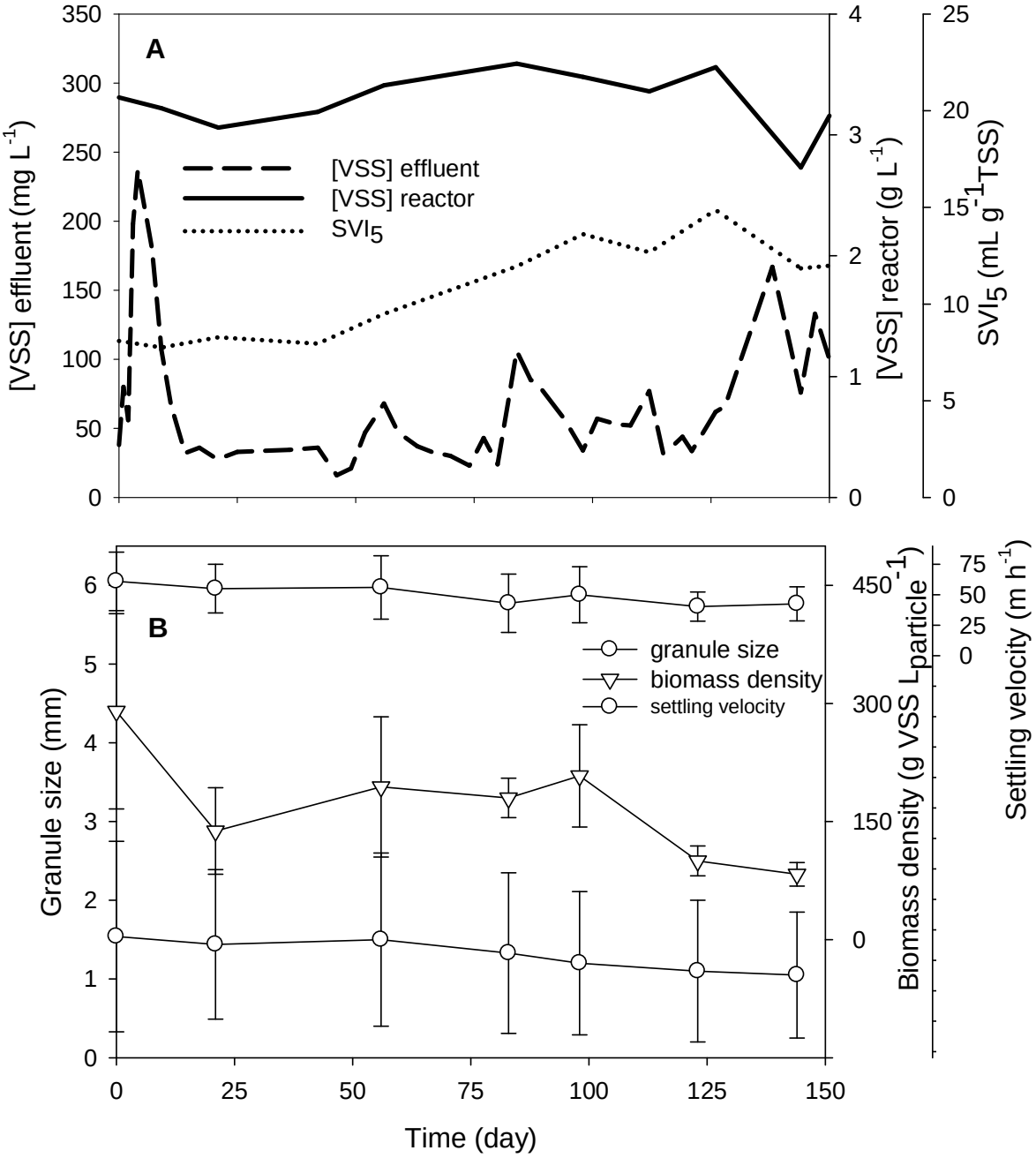

Figure 6.6 The aerobic granular sludge characteristics in term of; (A) volatile suspended solid (VSS) concentrations (reactor and effluent) and SVI_5 values; (B) granule size, biomass density and settling velocity.

6.3.3 Biomass characterization

The characteristics of aerobic granular sludge during the whole operational period were followed in terms of, SVI_5, granule size, settling velocity and biofilm density (Figure 6.6). Also, the EPS contents were monitored (Table 6.3). The results showed that the aerobic granular sludge employed and developed in this work possessed a good settling ability, even in front of shock load events as shown by the excellent settling velocities (40-60 m h^{-1}), SVI_5 (7-14 mL g^{-1}) and SVI_5/SVI_{30} (1.0) depicted on Figures 6.6A and 6.6B, respectively. In our results, only SVI_5 was depicted in Figure 6.6A since SVI_{30} values were identical to SVI_5 values in the whole experimental period, and the ratio SVI_{30}/SVI_5 was always one. The values obtained are in the range expected for granular sludge, according the review performed by (Gao et al., 2011).

The granule size decreased from its initial size, c.a. 1.5 mm to 1.0 mm at the end of the experimental period. In reviewing the literature, no data was reported on the impact of phenolic compounds over nitrifying granules morphology; however, the average size of granules obtained at the end of this study was into the same range reported by other studies: 0.3 to 1.6 mm for nitrifying granules (Mosquera-Corral et al., 2005) and 0.3 to 1.8 mm for phenolic-compounds-fed aerobic granules (Jiang et al., 2010; Suja et al., 2012). The biofilm density was decreased from its initial value, c.a. 290 to 80 g VSS $L_{particle}^{-1}$ at the end of the experimental period (Figure 6.6B). The decrease may be linked to the incipient presence of PNP-degraders in the granular sludge (bioaugmentation) since heterotrophic biofilms have lower biofilm densities than autotrophs (Charackalis and Marshall, 1990). The progressively increased of *o*-cresol loading rate applied in the whole operation period had also promoted the growth of the heterotrophic fraction and consequently affected the biofilm density through its present. As suggested by our results, the heterotrophs growth over nitrifying granules promoted by the *o*-cresol presence has altered the granule density from higher values to lower ones. Jiang et al. (2004) found similar results, i.e. density of granules decreased, and SVI values increased when a high phenol-loading-rate was applied.

EPS content in the granular sludge was monitored by determination of proteins (PN) and polysaccharides (PS) (Table 6.3). PS was the dominant compound of the EPS inside the granule, with PS/PN ratios always higher than 1.0 (Table 6.3). The concentration of PS increased during the first 25 days and later, remaining rather steady between 30 and 70 mg g^{-1} VSS. The PN content was rather stable between 7 and 17 mg g^{-1} VSS throughout the whole experimental period. It seems during the whole experimental period, PS was significantly impacted than PN. We postulate that the production of PS was stimulated by feeding *o*-cresol into the reactor and hence, promoted heterotrophs growth that is capable to produce PS more than nitrifiers. Similar finding was also reported by Yang et al. (2005) who observed that the increase of PS in the aerobic granules is dependent on the respiration activity of heterotrophic bacteria present in the aerobic granules i.e., a high catabolic activity favours the production of PS. Laudelout et al. (1968) further reported that nitrifying bacteria cannot utilize organic carbon for microbial growth and only 11% to 27% of energy generated goes to biosynthesis. It seems reasonable to conclude that the production of PS in nitrifying granules is strongly related to the fraction of heterotrophs present in the granules. Above all, despite the changed of granular characteristics during the simultaneous partial nitritation and *o*-cresol removal, the performance of the granular sludge reactor stably maintained. This finding indicates that the ability of granular sludge in term of contaminants

biodegradation is unaffected in response to the changed of granular characteristics. However, further research in a long –term operation need to carry out in order to elucidate the performance of the reactor in respond to the changed of granular sludge characteristics.

Table 6.3 Extracellular polymeric substances (EPS) content of aerobic granular sludge during the simultaneous partial nitritation and *o*-cresol biodegradation.

Period (day)	Polysaccharides (PS) (mg g^{-1} VSS)	Protein (PN) (mg g^{-1} VSS)	Ratio PS/PN
0	27 ± 2	11.2 ± 0.8	2.4
21	164 ± 7	10.9 ± 0.2	15.0
56	98 ± 4	13.6 ± 0.7	7.2
80	31 ± 9	6 ± 2	5.2
98	72 ± 5	5 ± 3	14.4
123	20 ± 2	5.1 ± 0.4	3.9
143	26 ± 2	12 ± 4	2.2

6.3.4 Identification of dominant species by FISH

At the end of the experimental period, samples of granular biomass were fixed, sliced, and further hybridised and analysed using FISH technique. All of the possible bacteria species present in the biomass were stained using probes listed in Table 6.2. The results from the FISH technique identified on one hand, that only *Acinetobacter* genus (Figure 6.7D) and β-AOB (Figure 6.7C) were the populations with higher occurrence, whereas *Nitrobacter* sp. (Figure 6.7B) was also detected, but at a very low occurrence. On the other hand, *Arthrobacter* sp. was not detected in the samples. The *Acinetobacter* genus is believed to be the responsible for *o*-cresol biodegradation; meanwhile, β-AOB would be responsible of the nitritation process. As can be seen, *Acinetobacter* genus tends to locate at the outer layer (Figure 6.7D), in fact, some filaments are visible as in Figure 6.4, and β-AOB tends to locate at the inner layers, although some are also located at the outer core (Figure 6.7C). Therefore, seems that the hypothesis of microbial stratification following different metabolic activities could be plausible.

Acinetobacter genus detected in our biomass was also observed by Lee et al. (2011) and corroborates with one of the bacteria population dominant in the bioaugmented PNP-degraders (Pramparo et al., 2012). According to these researchers, in that biomass, the *Arthrobacter* sp. fraction accounted for 30 ± 13% of the total bacteria, whereas genus the *Acinetobacter* fraction was 31 ± 15% (Pramparo et al. 2012). Adav et al. (2007) reported that an *Acinetobacter* strain isolated from phenol-degrading aerobic granules exhibited a high propensity to attach to a solid surface. In this sense, our results seems to indicate that only *Acinetobacter* genus and not *Arthrobacter* sp. was capable of being retained in the granular airlift reactor, maybe because the former have better auto-aggregation properties than the later. More specific studies are needed to confirm this extreme.

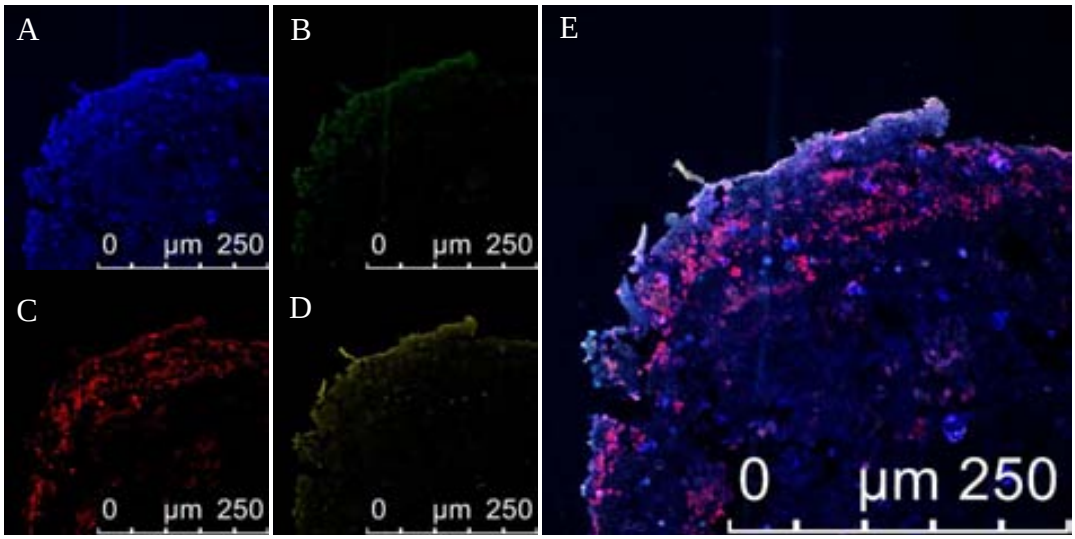

Figure 6.6 FISH image of a sliced granule collected at the end of the experimental period (Bar = 250 µm). A) Blue: all bacteria (UNIV1390 and EUBmix); B) green: *Nitrobacter* sp. (NIT3); C) red: β-AOB (Nso190); D) yellow: *Acinetobacter* genus (ACA652) and E) Merge Image. Centre of the granules is on the bottom right corner.

6.4 Conclusions

In this study, the simultaneous partial nitritation and *o*-cresol biodegradation was successfully accomplished in a single reactor with aerobic granular biomass. An effluent for a subsequent Anammox process was stably maintained for more than 100 days. Bioaugmentation strategy was enhanced the formation of granular sludge with a special architecture in which an external layer of heterotrophs was completely degraded *o*-cresol and at the same time, this external shell is protecting ammonia oxidising bacteria (AOB) against this potentially inhibitory compound and the *o*-cresol shock load events. Biomass characterization indicated that the wastewater containing *o*-cresol had a minor impact on the granules size, density, PS/PN ratio and settling velocity. This suggests that continuous aerobic granular sludge reactors are a promising technology for simultaneous removal of high-strength ammonium wastewaters containing recalcitrant compounds.

6.5 References

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Chapter 7

Performance of an aerobic granular reactor performing simultaneous partial nitritation and *o*-cresol biodegradation in front of sequentially alternating pollutant (SAP) scenarios

Summary

Often, the industrial production processes change periodically, leading to variable characteristics of the wastewater in terms of concentrations, composition and perhaps even environmental conditions. Hence, the wastewater treatment plant (WWTP) installed in the industrial sector must be robust, flexible and able to function effectively during these transient-state conditions. In this study, the performance of an aerobic granular reactor (AGR) performing simultaneous partial nitritation and *o*-cresol biodegradation under sequentially alternating pollutant (SAP) scenarios was investigated. Three SAP scenarios were designed and imposed during the continuous operation of AGR. The regular wastewater composition contained a high-strength concentration of ammonium (950 mg N L^{-1}) and *o*-cresol (100 mg L^{-1}). In each one of the SAP scenarios, 15 mg L^{-1} of the secondary phenolic compounds (i.e. *p*-nitrophenol (PNP), phenol and 2-chlorophenol (2CP)) were added in the regular influent for a short period of time (between 20 and 25 days). The results show that the performance of partial nitritation and *o*-cresol biodegradation were stably maintained and were not inhibited by the presence of PNP or phenol. However, the 2CP presence inhibited 90 % of partial nitritation and 25 % of *o*-cresol degradation rate within three days. This finding suggests that the ammonia oxidizing bacteria (AOB) is more sensitive to 2CP inhibition than heterotrophs (*o*-cresol-degraders). After the three SAP scenarios good efficiencies in the degradation of *o*-cresol (the primary phenolic compound) and nitritation were fully recovered in only 10 weeks. These findings show that the application of aerobic granular biomass to treat complex industrial wastewaters with highly variable influent composition is feasible.

7.1 Introduction

The industrial sector often produces complex wastewater containing inorganic and organic pollutants that are difficult to be treated. For instance chemical, coke, petrochemicals and refineries industries are producing wastewaters containing ammonium and several phenolic compounds (Chang et al., 2008; Diya'uddeen et al., 2011). Often, these effluents are being treated by physic-chemical means, such as, absorption, incineration and advanced oxidation process (Ahmaruzzaman, 2008). However, the treatment of these effluents is partial and costly since the solution used (physic-chemical processes) does not fully remove the contaminants and in certain cases, generate other toxic wastes. Thus, it is obvious that these methods are not fully efficient, expensive and non-sustainable. Biological treatment could be a potential solution since it provides a complete contaminants biodegradation, and it is associated with low investment and operation costs. Nevertheless, the (chemical) industry frequently is reluctant to use fully biological treatments. The interruption of production processes, varying production schedules and plant shut down due to maintenance purposes are among the dilemmas that industry encounter to not completely trust on a biological treatment. Often, the conventional biological treatment may become complex when the production processes change periodically, leading to changing characteristics of the wastewater in term of concentrations, composition and perhaps even environmental conditions (Sipma et al., 2010). Moreover, habitual scenarios without wastewater production (short and long term starvation for microorganisms), sequentially alternating pollutants (SAP) or shock loading episodes are seen and experienced by the industry.

The sole use of an advanced biological technology to treat thoroughly complex industrial wastewaters associated with inflow dynamic changes could be a feasible alternative. Among the novel advanced biological technologies available for wastewater treatment, the most attractive are the systems based on aerobic granular biomass (Adav et al., 2008a). Aerobic granular biomass has proven feasible, resistant and resilient to cope with dynamic changes in wastewater compositions or shock load events compare with suspended biomass (Jiang et al., 2010; Maszenan et al., 2011). Additionally, in such systems, the biomass grows as compact and dense microbial granules, allowing greater biomass retention in the reactor with attractive impact on increasing substrate capabilities (Adav et al, 2008a). The granular sludge posses a unique structure assembled by consortia of microbes wherein the various species perform different and specific roles in biodegradation of contaminants during wastewater treatment (Beun et al., 1999). Close association among the microbial entities and degradation of contaminants via multiple steps result in the layered structures seen in aerobic granules (Li et al., 2008). This layered structure also creates concentration gradients of target compounds inside the granule and these protect microorganisms from the impact of direct acute toxicity associated with the compounds (Maszenan et al., 2011). The toxicity impact of compounds also could be reduced further by operating an aerobic granular biomass reactor in a continuous mode. Thus, the ability of aerobic granule to outperform during transient states is possible.

Among the episodes of transient-state conditions experienced by the industry, SAP scenarios have received little attention in studies for wastewater treatment (Sipma et

al., 2010). Several SAP scenarios are similar to starvation periods either partial or complete starvation (Jorge and Livingston, 2000; Emanuelsson et al., 2008; Osuna et al., 2008; Buitrón and Moreno-Andrade, 2011). Few studies have been focused on the effect of biodegradation process in the present of secondary compounds (Beristain-Cardoso et al., 2011; Silva et al., 2011; Pérez-González et al., 2012).

In the case of wastewaters simultaneously containing ammonium and phenolic compounds, few studies on nitrification process in the presence of phenolic compounds mixtures have been carried out with the aim to remove all contaminants simultaneously (Silva et al., 2011; Pérez-González et al., 2012). For wastewaters containing a high-strength ammonium concentration, partial nitritation is the preferred biological treatment compared to nitrification process. The former process offers significant savings in aeration requirement than the latter process. The feasibility of simultaneous nitritation and phenolic compounds removal was already demonstrated in Chapter 6 and 8. In these chapters, simultaneous ammonium and *o*-cresol or *p*-nitrophenol (PNP) removal was successfully demonstrated. However, the biodegradation study only involved a wastewater containing ammonium and an individual phenolic compound. Industrial wastewaters are highly heterogeneous and may contain a multiple phenolic compounds and ammonium. Thus, the research of simultaneous ammonium and phenolic compounds removal using a continuous aerobic granular reactor should be extended aiming to investigate the feasibility of this bioremediation in the presence of secondary phenolic compounds or under transient-state conditions, i.e. SAP events. This research will explore the potential of granular sludge to remediate complex industrial wastewaters and its feasibility to cope with transient-state conditions.

Therefore, the goal of this study is to evaluate the effects of SAP scenarios in an aerobic granular reactor (AGR) performing simultaneously partial nitritation and *o*-cresol removal operating in continuous mode. Three SAP scenarios were designed to mimic the situation of variable production schedules, encountered in the industry and resulted in the presence of secondary phenolic compounds in regular influent. In each of the SAP scenarios, a model wastewater containing regular influent (ammonium and *o*-cresol) in the presence of one of the following phenolic compounds i) *p*-nitrophenol (PNP) ii) phenol iii) 2-chlorophenol (2CP) was fed to the bioreactor. The performance of the AGR was evaluated accordingly.

7.2 Materials and Methods

7.2.1 Chemicals and reagents

o-Cresol, 2-chlorophenol (2CP) in a concentrated solution (purity 99%) and *p*-nitrophenol (PNP) in a granular solid form (purity 99%) were employed and supplied by Panreac (Spain. Ammonium chloride (purity 99.5%) was supplied by Carl Roth (Germany). Phenol and all the others chemicals and reagents were purchased from Sigma-Aldrich (Spain) and the highest purities available were employed.

7.2.2 Experimental set-up

A glass airlift reactor with a working volume of 2.6 L was used 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. 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). Feeding to the reactor was made with a membrane pump (ProMinent Gamma/L). DO in the reactor was regulated by rotameter (Aalborg, USA).

7.2.3 Reactor operation and wastewater

The AGR treating wastewaters containing simultaneously ammonium and *o*-cresol was utilized. The reactor was continuously operated for a period of 5 months and was fed with a synthetic influent containing a high-strength ammonium concentration of 950 ± 25 mg $N\text{-}NH_4^+$ L^{-1},an *o*-cresol concentration of 100 ± 5 mg 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. Prior to the SAP scenarios study, the reactor was performing stable partial nitritation with average effluent concentrations of: 400 ± 27 mg $N\text{-}NH_4^+$ L^{-1}, 561 ± 25 mg $N\text{-}NO_2^-$ L^{-1}), 0.5 ± 0.3 mg $N\text{-}NO_3^-$ and complete and steady *o*-cresol removal during the whole period. In this experimental study, the temperature of the reactor was kept at 30 ± 1.0 ^{o} C and DO was maintained at 2 mg O_2 L^{-1}. The pH of the reactor was maintained at 8.1 ± 0.4 by a regular addition of $NaHCO_3$ into the reactor.

7.2.4 Description of the sequentially alternating pollutant (SAP) scenarios

Three scenarios of sequentially alternating pollutant (SAP) were designed to mimic variable production schedules commonly faced by the industries. In each scenario, a secondary compound was entering to the AGR for a short period of time (usually between 20 and 25 days). The usual influent composed of ammonium and *o*-cresol was kept in all of the scenarios. Three phenolic compounds namely, PNP, phenol and 2CP were selected. The SAP scenarios imposed to the reactor are represented in Figure 8.1, and the corresponding influent compositions in each SAP scenario are detailed in Table 7.1. Briefly, in a SAP scenario, the bioreactor treating the regular influent suddenly suffers the addition of a secondary phenolic compound (due to a change in the production schedule) to the influent for a period of 2 to 3 weeks. After that period, the production returns to its normal schedule and consequently, the bioreactor switch back to treat its regular influent. Note that, the three experiments were carried out continuously from one SAP to another.

7.2.5 Analytical methods

o-Cresol, PNP, phenol and 2CP were determined by High Performance Liquid Chromatography (HPLC) (UltiMate 3000, Dionex Corporation) using an Agilent Zorbax SB-C18 (4.6 x 100 mm, 3.5 μm) column and a UV detector set at 254 nm, the flow rate was 1.875 mL min^{-1} and the column temperature was maintained at 30 ºC (Pramparo et al., 2012). The mobile phases were ultrapure water containing H_2SO_4 at pH 1.41 and HPLC-grade methanol following a gradient elution. The gradient started from 100% of acidified water and progressively changed to 50:50 v/v of water:methanol in 18 min, then it remained isocratic until 20 min. The injection volume was 20 μL and the maximum pressure in the column was approximately 290 bars. The ammonium concentration measured as total ammonia nitrogen (TAN) was analyzed using a continuous flow analyzer based on potentiometric determination. The nitrite and nitrate were measured with ionic chromatography (ICS-2000 Integrated Reagent-Free IC System, DIONEX). 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 term of size, granule density and settling velocity. The size distribution of the granules was measured 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 extracellular polymeric substances (EPS) were extracted from the granules using formaldehyde + NaOH according to Adav and Lee (2011). In brief, 50 mL of sample from the reactor was taken, and the granules were left to settle. Supernatant was discarded, and the granules were washed with mili-Q water. The granules were crushed using a mortar and pestle. 10 mL of the crushed granules sample was added with 0.06 % formaldehyde. The sample was kept in 4 °C for 1 hour. Then, 4 mL of NaOH (1M) was added into the sample and kept in 4 °C for 3 hours. Later, the supernatant from the sample was taken and centrifuged at 4 °C, 10 000 rpm and 30 minutes. The EPS extracted sample (supernatant) was recovered and further analysed for polysaccharides and protein contents. The polysaccharides content in the EPS extracted sample was determined using a calorimetric method with glucose as the standard (Dubois et al., 1956). The protein content in the extracted sample was measured using the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951).

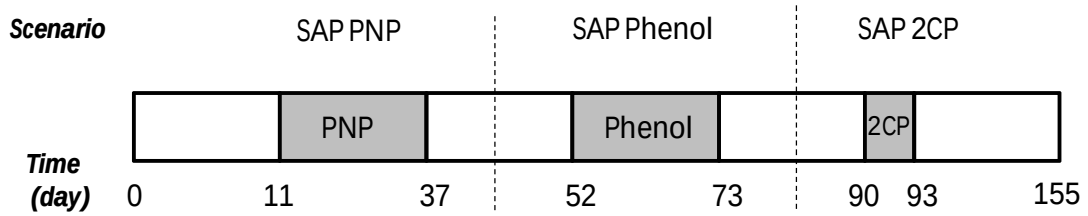

Figure 7.1 A Simplified diagram of sequentially alternating pollutant (SAP) scenarios imposed to the aerobic granular reactor performing simultaneous partial nitritation and *o*-cresol removal operating in continuous mode. The presence of the secondary phenolic compound is also illustrated; PNP (*p*-nitrophenol), phenol and 2CP (2-chlorophenol).

Table 7.1 Feeding compositions imposed during the continuous operation of aerobic granular sludge reactor under sequentially alternating pollutant (SAP) scenarios study. Concentration of each substrate involved in the SAP study is ammonium, 950 mg N-NH_4^+ L^{-1}; *o*-cresol, 100 mg L^{-1}; *p*-nitrophenol (PNP), 15 mg L^{-1}; phenol, 15 mg L^{-1}; 2-chlorophenol (2CP), 15 mg L^{-1}.

Time (days)	Substrate in the influent	Period	Sequentially Alternating Pollulant Scenarios
0-10	N-NH_4^+ + *o*-cresol	I	
11-36	N-NH_4^+ + *o*-cresol + PNP	II	SAP PNP
37-45	N-NH_4^+ + *o*-cresol	III	
46-51	N-NH_4^+ + *o*-cresol	IV	
52-72	N-NH_4^+ + *o*-cresol + phenol	V	SAP Phenol
73-80	N-NH_4^+ + *o*-cresol	VI	
81-89	N-NH_4^+ + *o*-cresol	VII	
90-92	N-NH_4^+ + *o*-cresol + 2CP	VIII	
93-107	N-NH_4^+ [a]	IX	SAP 2CP
108-131	N-NH_4^+ [b]	X	
132-155	N-NH_4^+ + *o*-cresol	XII	

[a]reactor operated in batch mode due to failure caused by 2CP addition
[b]reactor resumed in continuous mode

7.3 Results and discussion

7.3.1 Effect of PNP as secondary phenolic compound

In the SAP PNP scenario, between day 11 and 37, the reactor was fed with a regular influent (ammonium and *o*-cresol) and a secondary phenolic compound, PNP. After day 37, the influent returned to its normal feeding composition. Figure 7.2 illustrates the results obtained during the SAP PNP study. The results clearly indicate that the partial nitritation was stably maintained during and after the presence of PNP (Figure 7.2B). Moreover, it was observed that in the second half of SAP PNP event, the ammonium oxidation into nitrite was slightly increased. Consequently, the nitrite/ammonium ratio was also affected and shifted from 1.5 to 2.0 (Figure 7.2A).

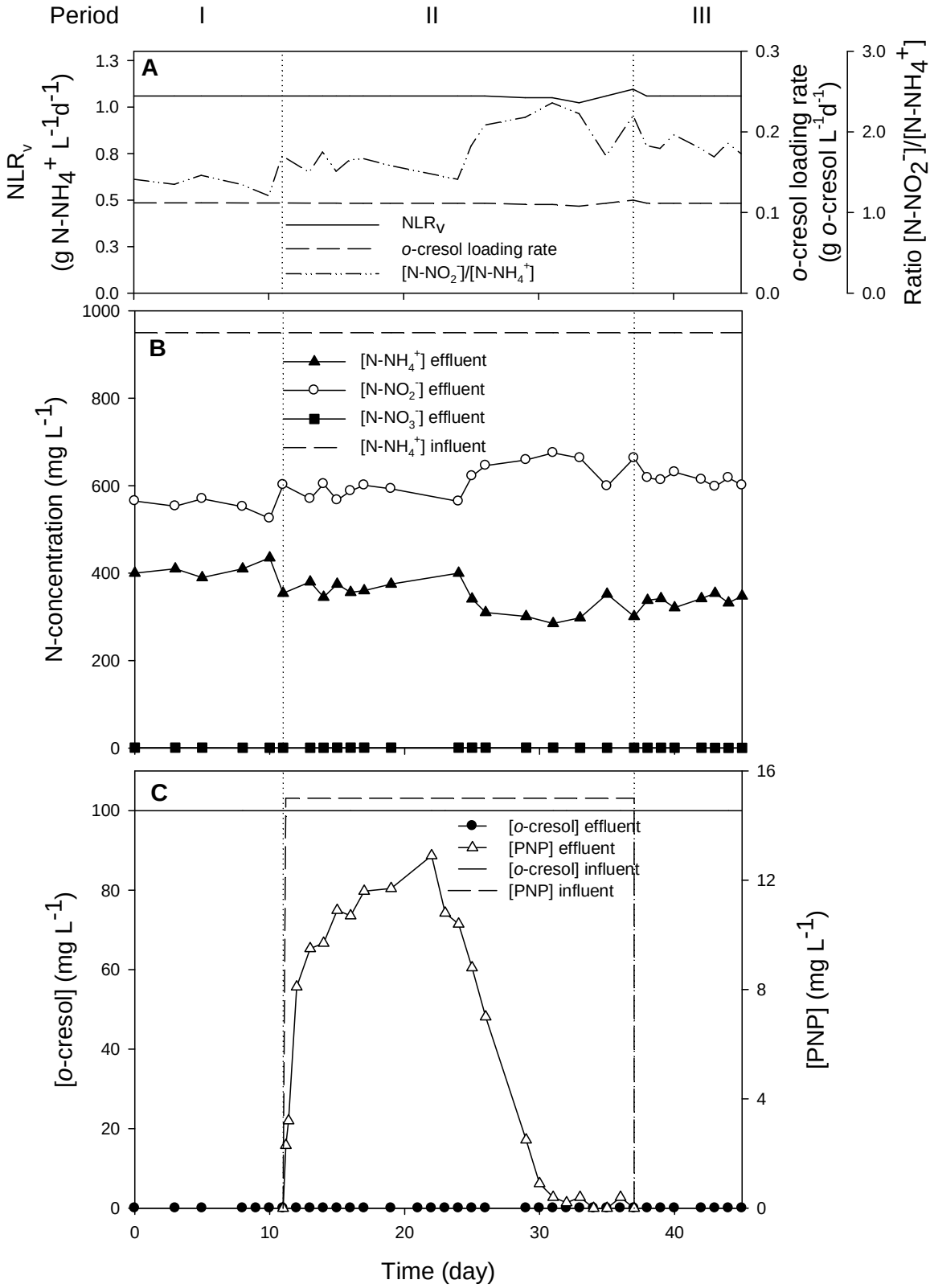

Figure 7.2 Performance of the aerobic granular reactor treating simultaneously ammonium and *o*-cresol under sequentially alternating pollutant (SAP) scenario study with *p*-nitrophenol (PNP). (A) Volumetric nitrogen loading rate (NLR_v), volumetric *o*-cresol loading rate and [N-NO_2^-]/[N-NH_4^+] ratio. (B) Nitritation performance. (C) Removal performance of *o*-cresol and PNP.

The increase of ammonium oxidation rate coincident immediately after the PNP started to degrade (Figure 7.2C). An increase of 10 percent of ammonium oxidation rate could be due to the PNP inhibitory impact over AOB has ended. The PNP inhibitory impact over AOB was pronounced when the PNP accumulation started to surpass beyond 10 mg PNP L^{-1} on day-19 until day-23. During these days, ammonium oxidation rate was slightly decreased. In the second half of the SAP PNP event, immediately after the PNP started to degrade, a noticeable increase of ammonium oxidation rate was observed. This observation verified that the presence of PNP in the wastewater has an inhibitory impact over AOB, dependent on the PNP concentrations. These observations were further confirmed by the results obtained in Chapter 8, where one of the possible reasons behind the unstable PNP degradation performance was an inhibitory impact of PNP over AOB. Even more, the reason was supported by the estimated value of the PNP inhibition coefficient, K_i over nitritation that was 7 ± 2 mg L^{-1}. Several studies also reported that PNP are an inhibitory substrate to nitrifiers, for instance, Suja et al. (2012) observed that nitrification rate was decreased when 50 mg PNP L^{-1} was fed as a sole carbon source during the cultivation of PNP- aerobic granules in a SBR reactor. Goh et al. (2009) reported that the ammonium removal was deteriorated to 48 % due to the inhibitory effect caused by an addition of 150 mg PNP L^{-1} to activated sludge in the SBR reactor.

Furthermore, the *o*-cresol biodegradation was unaffected and always maintained a complete *o*-cresol removal in the presence of PNP and after SAP PNP event (Figure 7.2C). On the contrary, PNP fed into the reactor was accumulated up to 13 mg L^{-1} in the first 10 days of its imposed feeding. Later, a complete PNP removal was achieved within a week and maintained until the SAP PNP ended. The PNP accumulation incidence in the first 10 days of SAP PNP event could be possibly related to the acclimation phase of heterotrophic bacteria able to degrade PNP. Interestingly, no inhibition impact of PNP over the *o*-cresol removal was observed during the incidence. After that accumulation period, the PNP was consumed by heterotrophic bacteria able to degrade PNP, thus a complete PNP removal was accomplished. The obtained results corroborate with study reported by Pramparo et al. (2012) and Martín-Hernández et al. (2012) whose observed that PNP-degrading activated sludge was able to consume several phenolic compounds as the sole carbon source. Those authors reported that the PNP-degrading activated sludge used in their study was formed by several microbial species. Studies of microbial consortium bacteria able to degrade several phenolic compounds have also been reported (Yi et al., 2006; Saravanan et al., 2008; Buitrón and Moreno-Andrade, 2011; Lee et al., 2011). In addition, Saravanan et al. (2008) and Lee et al. (2011) reported that the degradation of phenolic compounds was consumed by predominant microbial species present in the biomass. For the starting-up of this reactor treating ammonium and *o*-cresol simultaneously, it was bioaugmented with a PNP-degrading activated sludge (Chapter 6). Moreover, in Chapter 6, it also observed that the predominant microbial species in the PNP-degrading activated sludge remained present in the reactor after stable and long term operation of *o*-cresol removal was achieved. Thus, degradation of PNP by the heterotrophic bacteria able to degrade PNP was expected during SAP PNP event. However, a lag phase on the PNP degradation was observed in the first 10 days of the SAP PNP event and this is possibly due to the acclimation period of specific heterotrophic bacteria to consume PNP as mentioned previously. It seems like the *o*-cresol and PNP degradation was degraded by different types of heterotrophic bacteria

present in the biomass. During a long-term operation of *o*-cresol degradation, the heterotrophic bacteria able to degrade *o*-cresol was outgrowth and the heterotrophic bacteria able to degrade PNP was dormant. When PNP was available, the later bacteria began to synthesis an enzyme to be able to consume PNP and this bioprocess requires time, thus a lag phase was observed prior the PNP degradation. Several studies reported a shift in microbial communities during SAP feeding containing recalcitrant compounds, for instance, Emanuelsson et al. (2008) and Osuna et al. (2008). These authors observed a specific microbial community present to biodegrade a specific recalcitrant compound, and when the feeding substrate (recalcitrant) alternating interchange, the dominant microbial community was also shifted accordingly. Thus, further research should be done to investigate and understand the microbial dynamics of simultaneous nitritation and phenolic compounds removal using an aerobic granular reactor under SAP scenarios by using a suitable microbial identification technique.

Regarding the biomass evolution, during the SAP PNP event, a slight increase in biomass concentration in the reactor (VSS_R) was observed (Figure 7.5). Tomei et al. (2006) observed the PNP degrader growth rate increased with decreasing of PNP concentration as the sole carbon source. At high PNP concentration > 80 mg PNP L^{-1}, the authors explained that the decrease of growth rate was due to the PNP inhibitory impact. In the present study, a low PNP concentration was fed into the reactor; thus a positive biomass growth could be promoted. The increase of VSS_R in the present study also could be linked to the notable increase of EPS contents in the granules during the SAP PNP event (Table 7.2). The trend of VSS_R and EPS in the SAP PNP event was almost similar. Moreover, the biomass concentration in the effluent (VSS_E) was low and stable at ca. 50 mg L^{-1}during and after the SAP PNP event. This stability is a clear sign of stable morphology of granular sludge. The low VSS_E observed could be possibly due to the resilient of aerobic granular biomass to the PNP inhibitory impact, thus resulting in a low biomass detachment rate. In addition, the stability of aerobic granules was also exhibited by the low and stable SVI_5 during and after the SAP event.

7.3.2 Effect of phenol as secondary phenolic compound

Upon completion of the SAP PNP scenario, the reactor was maintained and fed with regular influent for about 2 weeks. Then, the SAP phenol was carried out with the presence of secondary phenolic compound of 15 mg phenol L^{-1} in the regular influent fed into the reactor. Figure 7.3 illustrates the results obtained from the study of SAP phenol. The partial nitritation was stably maintained during the presence of phenol and after the SAP phenol event. The partial nitritation was successfully sustained without inhibition by phenol during the SAP phenol event. Moreover, the volumetric nitrogen loading rate (NLR_v) and nitrite/ammonium ratio were retained at around 1.2 g $N\text{-}NH_4^+$ $L^{-1}d^{-1}$ and 1.3, respectively. The results suggest that partial nitritation process was not inhibited by the presence of phenol at a concentration of 15 mg L^{-1}. Several studies reported the tolerance of nitrification in the presence of high phenol concentration i.e > 300 mg L^{-1} (Amor et al., 2005; Yamagishi et al., 2001; Silva et al., 2011; Pérez-González et al., 2012). In fact, these authors also observed ammonium and phenol was simultaneously biodegraded. Nevertheless, studies on inhibitory effect of phenol over nitrification at low phenol concentration < 15 mg L^{-1} were also

reported (Liu et al., 2005; Lauchnor et al., 2011). Liu et al. (2005) observed that using nitrifying bacteria embedded in granules, the nitrate productivity could be recovered to 77% and 67% in the presence of 15 and 20 mg phenol L^{-1}, respectively. Nevertheless, these authors also reported that nitrifying activity was recovered completely after degradation of phenol when initial phenol concentrations were not higher than 10 mg L^{-1}. Similarly, Lauchnor et al. (2011) reported that the ammonium oxidation rate in the biofilms was inhibited 50 % when exposed to 5 mg phenol L^{-1}, a lower phenol concentration compared to Liu et al. (2005). Contrary, in the present study by utilizing aerobic granular biomass composed of nitrifiers and heterotrophs, it was showed that partial nitritation was unaffected, resilient and could withstand with the possible inhibition by phenol. Possible explanations for the tolerance of partial nitritation to phenol inhibition in the present study are i) diffusion limitation of inhibiting substrates inside the biofilm and ii) biodegradation of phenol by heterotrophic bacteria most likely at the granule surface.

With regard to the *o*-cresol degradation, a complete removal of *o*-cresol was maintained during and after the SAP phenol event. Simultaneously, phenol was completely removed. The presence of phenol in the reactor was not inhibited the *o*-cresol degradation process. This finding corroborates with the result obtained by Kar et al. (1997) who reported that the *o*-cresol biodegradation rate was not affected by the presence of phenol.

In the present study, the simultaneous removal of *o*-cresol and phenol was mainly linked to the ability of *o*-cresol degrading bacteria to degrade phenol. Simultaneous removal of cresols and phenol by a predominant microbial species in a mixed culture was reported by several studies (Saravanan et al., 2008; Wang et al., 2009). For instance, Wang et al. (2009) reported that *Arthrobacter* sp. isolated from the sludge of a sewage plant was able to degrade phenol and *p*-cresol under high salt conditions, and several phenolic compounds as the sole carbon source. Similarly, Saravanan et al. (2008) reported a mixed culture predominantly of *Pseudomonas* sp. able to degrade both, phenol and *m*-cresol. They also observed that the presence of phenol below 300 mg L^{-1} did not inhibit *m*-cresol degradation and moreover, phenol was degraded preferentially and earlier than *m*-cresol and it did not show any lag phase in its biodegradation. Saravanan et al. (2008) explained that the preferential uptake of phenol over *m*-cresol for given concentrations of the substrates, indicate that phenol compared to *m*-cresol is a much simpler carbon source, and therefore can be easily utilized by the mixed culture without any lag phase in its biodegradation. Besides, Lee at al. (2011) reported that the microbial species in phenol-acclimated aerobic granules remained dominant when *ortho*-, *meta*- or *para*-cresol were treated in batch experiments. Above all, it suggests that heterotrophic bacteria able to degrade *o*-cresol could also degrade phenol. It seems like phenol degradation is following the same metabolic pathway with *o*-cresol, where the non specific enzyme present in the biomass could oxidize both phenolic compounds *o*-cresol and phenol simultaneously. Masunaga et al. (1986) reported that the main degradation route of *o*-cresol was following the 3-methyl catechol pathway. Three-methyl catechol was further degraded through at least two meta cleavage pathways. At this stage, a common route for catechol oxidation in the metabolic pathway of phenol was also applied in the *o*-cresol degradation pathway (Ribbons, 1966; Buswell, 1975). It is very likely that the phenol degradation was consumed by other heterotrophic species

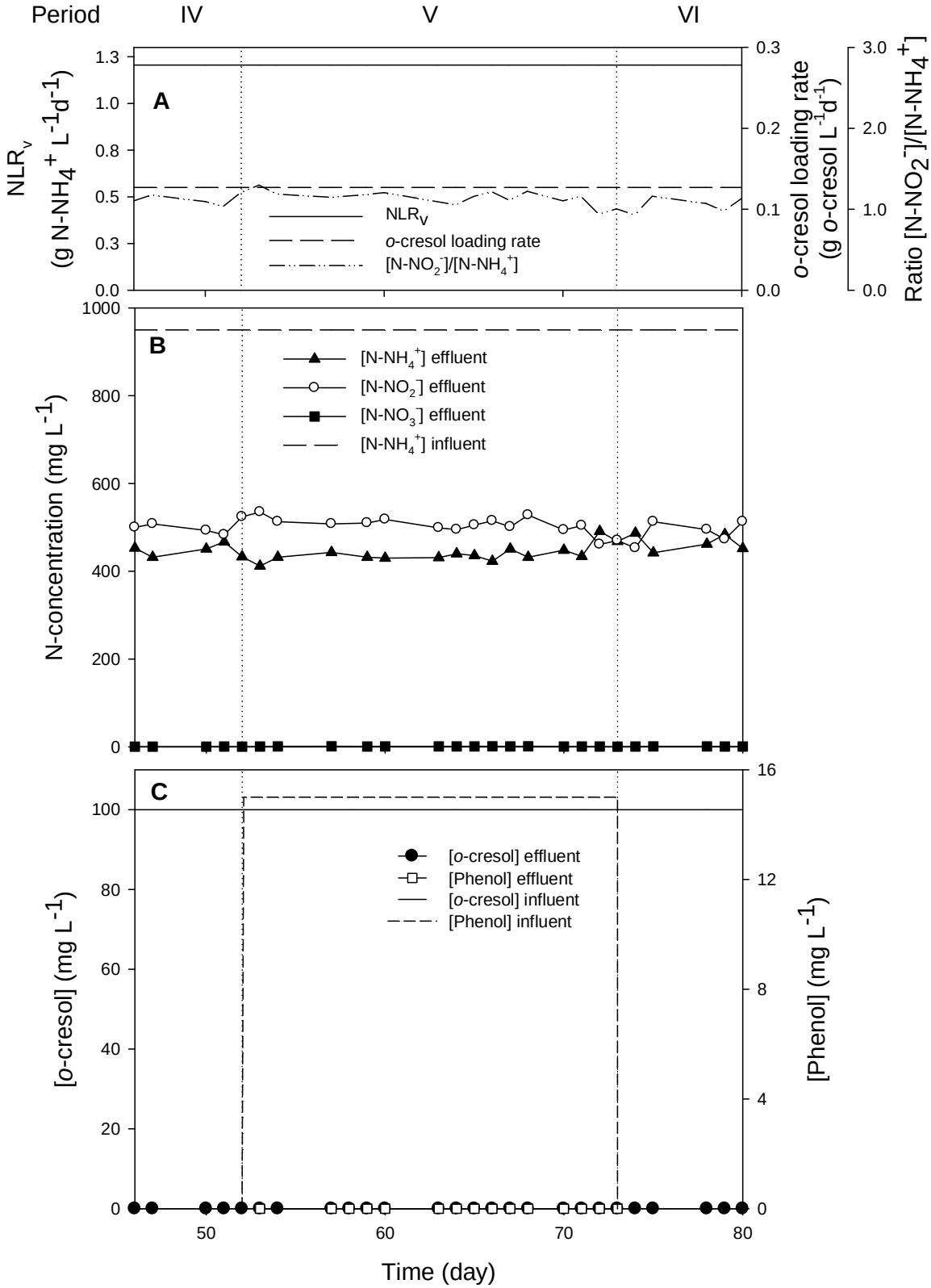

Figure 7.3 Performance of the aerobic granular reactor treating simultaneously ammonium and *o*-cresol under sequentially alternating pollutant (SAP) scenario study with phenol. (A) Volumetric nitrogen loading rate (NLR_v), volumetric *o*-cresol loading rate and [N-NO_2^-]/[N-NH_4^+] ratio. (B) Nitritation performance. (C) Removal performance of *o*-cresol and phenol.

since no lag phase was observed in its degradation (previously observed in the SAP PNP event) and phenol was degraded from the very beginning of the SAP phenol event.

In terms of biomass evolution, the VSS_R was decreased during the SAP phenol event (Figure 7.5). Consequently, a notable amount of biomass was washout from the reactor during the SAP event and reflected by the increase of VSS_E and the slight decrease of SVI_5. The decrease of VSS_R (and increase of VSS_E) could be mainly due to the unusual detachment event of biomass from granules linked to the growth of biomass. The presence of phenol has promoted the growth of the heterotrophic biomass. In general, the specific growth rate of heterotrophs is proportional to its decay rate. The decay activity of aerobic granules is linked to the biomass detachment. Thus, is expected that more biomass should be washout in the effluent due to the increase in heterotrophs growth rate and the biomass decay. Consequently, less biomass was retained in the reactor during this period as depicted in Figure 7.5. In any case, clearly, after the SAP phenol event, the biomass detachment returned to its normal behavior i.e. lower VSS_E values were recorded (Figure 7.5, day 73 until 89). Several studies reported that phenol was a preferable substrate than cresols and stimulated biomass growth. Saravanan et al. (2008) reported that phenol was a preference substrate than *m*-cresol, and this substate was degraded earlier by a mixed culture predominantly of *Pseudomonas* sp because phenol compared to *m*-cresol is a much simpler carbon source. These authors also reported that the high values of yield coefficients were obtained in their study, especially at lower concentrations of the substrate combinations (100 mg phenol L^{-1} and 100 mg *m*-cresol L^{-1}). However, the biomass yield decreased with an increase of substrate combinations (>100 mg phenol L^{-1} and > 100 mg *m*-cresol L^{-1}). Kar et al. (1997) found that the specific growth rate of *Artrobacter* spp. with phenol was higher than with *o*-cresol. The estimated specific growth rate was ca. 0.8 h^{-1} and 0.76 h^{-1} at the concentration of 400 mg L^{-1} with phenol and *o*-cresol, respectively.

Although a notable amount of biomass was detached from granules and washed out in the effluent during the SAP phenol event, the compositions of detached biomass could be dominated by EPS rather than by consortia of microbes. An aerobic granule is composed of microbial aggregates assembled by consortia of microbes, and the closed aggregation between bacterial cells is glued by extracellular polymeric substances (EPS) (Maszenan et al., 2011). EPS, mainly contains polysaccharide and protein, is known as a bioglue with a high polysaccharide content that could facilitate cell to cell interaction and further strengthen microbial structure through the formation of a polymeric matrix leading to aerobic granules (Adav et al 2008b). EPS also maintains the integrity of granules and protect microorganisms from the impact of direct acute toxicity associated with the compounds and against the harsh external environmental conditions (Maszenan et al., 2011). In this study, a strong evidence illustrated that EPS could be the major components in the VSS_E, in view of the fact that the simultaneous nitritation and *o*-cresol degradation was unaffected by the presence of phenol. Table 7.2 illustrates a noticeable decrease of the EPS during the SAP phenol scenario compared to the SAP PNP event. The EPS was extracted from the granules, and the decrease of EPS contents in the granules should correlate to the detached biomass from granules.

7.3.3 Effect of 2CP as secondary phenolic compound

In the SAP 2CP scenario, the regular influent and a secondary phenolic compound, 2CP were fed into the reactor for a short period of time. Figure 7.4 illustrates the results obtained from the SAP 2CP scenario study. In despite of the successful performances of previous scenarios, the results clearly show that in this case, the partial nitritation was inhibited by 2CP. During the SAP 2CP event, it took only 3 days for 2CP to inhibit about 90 % of ammonium oxidation activity. It is suggested that 2CP was exceptionally toxic to ammonium oxidizing bacteria (AOB), in spite of the use of granular sludge. Therefore, extreme precaution needs to be taken when dealing with wastewaters containing chlorophenols. The inhibitory impact of 2CP over nitrifiers has been reported in several studies. Satoh et al. (2005) evaluated the effect of 10 mg 2CP L^{-1} on nitrifying biofilm, and they observed that nitrification activity was significantly inhibited. Moreover, Martínez-Hernández et al. (2011) and Silva et al. (2011) reported that when nitrifying sludge was exposed to 5 and 50 mg C $L^{-1}$of 2CP, ammonium consumption was decreased to 10 % and 16 %, respectively.

Regarding the *o*-cresol degradation, as it could be expected, the degradation of *o*-cresol was inhibited during the presence of 2CP into the reactor (Figure 7.4C). Within 3 days, *o*-cresol was built-up at 25 mg L^{-1} which corresponded to 25 % inhibition of *o*-cresol degradation. In addition, 2CP was not consumed and accumulated at 10 mg L^{-1} in the same period. These findings suggest that 2CP is considerably toxic and also inhibitory to heterotrophs even the same granular biomass was used for the other SAP scenarios, and an eventual acclimatization to phenolic compounds was expected, in the sense that the granular sludge might have acquired some ability (an enzymatic level) for consuming 2CP, when the inoculum source was adapted to several phenolic compounds (*o*-cresol, PNP, phenol). Nonetheless, further works are necessary in order to understand this phenomenon. Considering that multiple biological processes (i.e. ammonium and *o*-cresol oxidation) were taking place in the present study, an influent containing 15 mg 2CP L^{-1} is classified as highly toxic and inhibitory. Therefore, to treat simultaneously a complex wastewater containing 2CP, an appropriate strategy must be imposed. The findings of the current study do not support the previous research on 2CP biodegradation. Silva et al. (2011) reported simultaneous removal of 2CP, *p*-cresol, phenol and *p*-hydroxybenzaldehyde in batch experiments using biomass acclimated with ammonium and *p*-cresol. Moreover, Pérez-Alfaro et al. (2013) reported that although the nitrifying sludge previously exposed to 2CP was unable to recover its ammonium and nitrite oxidation capacity completely, a complete 2CP consumption rate was achieved in all assays (tested 2CP concentrations were up to 10 mg C L^{-1}). Martínez-Hernández et al. (2011) also documented that despite nitrification rate was inhibited by 2CP, the nitrifying sludge was completely consumed 2CP in 30 days. Several studies also reported that degradation of chlorophenols could co-metabolic by the presence of growth substrates. Monsalvo et al. (2009) reported that cometabolic biodegradation of 4-chlorophenol by SBR reactors at different temperature achieved in the presence of phenol as growth substrate. Meanwhile, Tobajas et al. (2012) documented that the cometabolic biodegradation of 4-chlorophenol by *Comamonas testosterone* was enhanced with phenol and glucose as carbon sources. Nonetheless, the findings of the current study corroborate with Satoh et al. (2005) whose evaluated the effect of 2CP

(10 mg L^{-1}) on a nitrifying biofilm, finding both oxygen and nitrifying consumption rates were inhibited, and the 2CP was not consumed by the biofilm.

Considering that AOB was completely inhibited, the feeding containing 2CP was stopped, and the reactor was washed out, and the granular biomass was recovered. The new start-up of the reactor using recovered granular biomass was mainly focused on the recovery of i) nitritation and then, of ii) simultaneous partial nitritation and *o*-cresol removal. From day 93 until day 107, the reactor was operated in batch mode. Upon the recovery of ammonium oxidation activity, the reactor was switched back to continuous operation. The NLR_v was progressively increased until the partial nitritation producing an effluent suitable for a subsequent Anammox process was achieved (a similar effluent conditions before the SAP 2CP event). Once the steady state condition of partial nitritation was accomplished, the reactor was fed again with primary phenolic compound, *o*-cresol. It took 14 days for the partial nitritation process to start reactivated and 4 weeks for full recovery of partial nitritation. To the best of our knowledge, there are no studies reported the recovery of partial nitritation after exposure to phenolic compounds utilizing an aerobic granular sludge. The closest and comparable finding to our study are reported by Lim et al. (2012) whose documented a study on inhibitory impact of 2,4-dichlorophenol (2,4-DCP) on nitrogen removal in a sequencing batch reactor using activated sludge. They reported the nitrogen removal efficiency was recovered in 52 days when the introduction of 30 mg 2,4-DCP L^{-1} was stopped in the feeding. A considerably shorter recovery period in the present study (4 weeks) compared to Lim et al. (2012) was mainly due to aerobic granular sludge employed. Aerobic granular sludge is reported more tolerant, resistant and resilient from the impact of direct acute toxicity associated with the compounds, in this case, 2CP than activated sludge (Adav et al., 2008a; Maszenan et al., 2011). A limited penetration of 2CP into the biofilms inhibited only in the upper parts of biofilms, whereas the bacteria present in the deeper parts of the biofilms were still active (Satoh et al., 2005). Thus, a fast recovery of biological processes is expected. The results obtained in this work importantly evidenced that the nitritation process with aerobic granules could be reactivated and recovered after a short-term (ca. 3 days) exposure to toxic compounds.

Upon the recovery of partial nitritation process, immediately, a complete removal of *o*-cresol was recorded during the re-fed of *o*-cresol into the reactor (Figure 7.4C, see day 132). Within 4 days, the specific volumetric *o*-cresol loading rate (CLR_S) reached identical CLR_S prior to 2CP introduction i.e. at 0.04 g *o*-cresol VSS^{-1} L^{-1} d^{-1}. Considering only ammonium was fed into the reactor during the start-up and recovery of nitritation process, the *o*-cresol degrading bacteria were starved for a period of 40 days. The immediate recovery of *o*-cresol degradation was fast and noteworthy. A possible explanation of the fast *o*-cresol degradation might be that *o*-cresol degrading bacteria were only inhibited and unharmed by 2CP. Prior to substrate (*o*-cresol) availability, these bacteria survived in endogenous conditions and probably

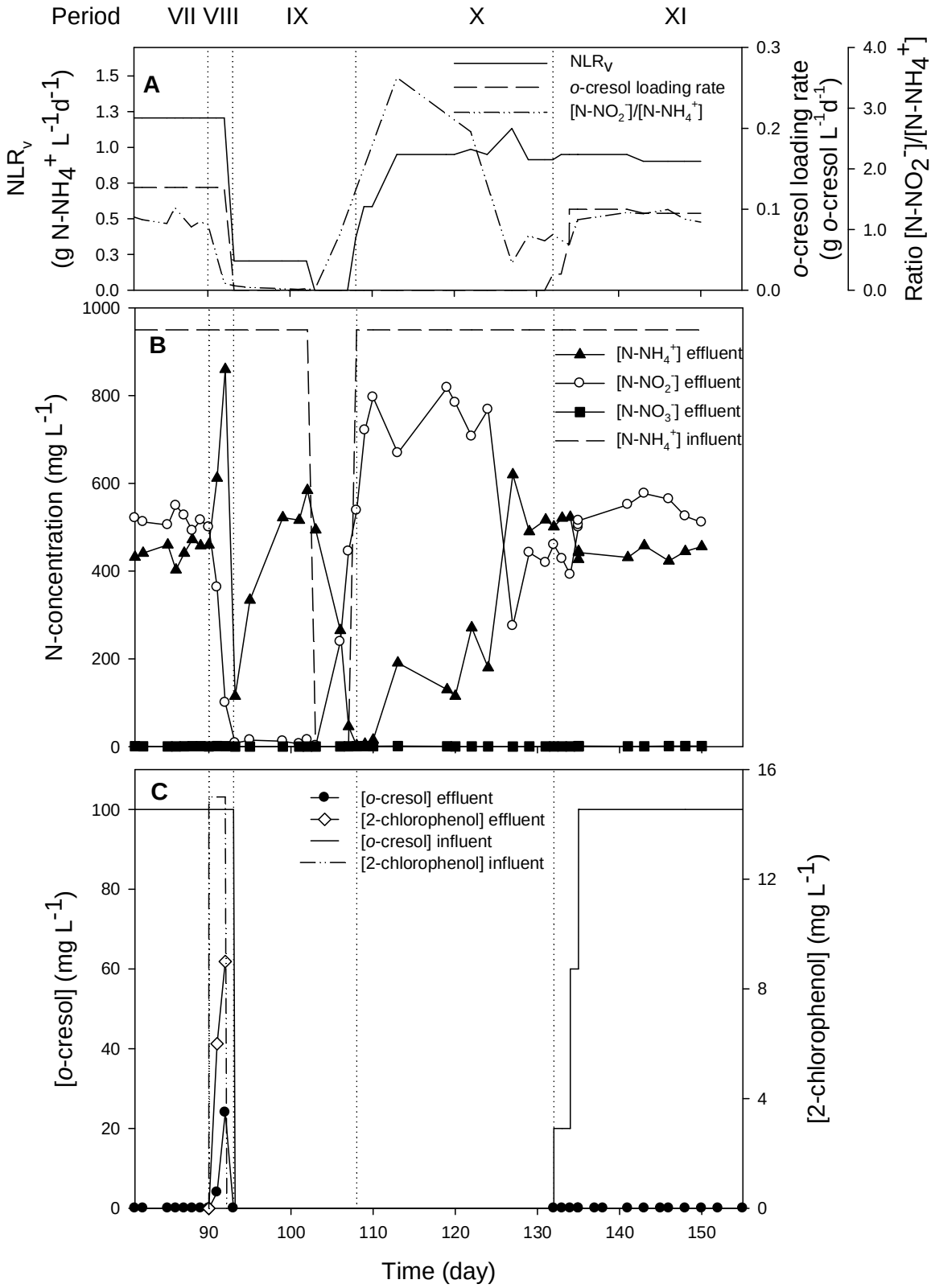

Figure 7.4 Performance of the aerobic granular reactor treating simultaneously ammonium and *o*-cresol under sequentially alternating pollutant (SAP) scenario study with 2-chlorophenol (2CP). (A) Volumetric nitrogen loading rate (NLR_v), volumetric *o*-cresol loading rate and [$N\text{-}NO_2^-$]/[$N\text{-}NH_4^+$] ratio. (B) Nitritation performance. (C) Removal performance of *o*-cresol and 2CP.

obtained carbon and nitrogen sources from cell lysis, ammonia and EPS (Wang et al., 2006). This is the first work showing the fast reactivation of aerobic granules treating *o*-cresol after a long-term starvation period coexisting with nitritation process. These findings also suggest that, in the simultaneous partial nitritation and *o*-cresol removal using aerobic granular biomass, a long-term starvation period does not affect *o*-cresol degrading bacteria. In addition, further investigation to monitor and understand the dynamic of microbial community in SAP scenarios using appropriate microbiological tools should be performed.

Evidently, due to inhibitory impact of 2CP, biomass detachment increased and consequently, the VSS_R reduced and biomass washout increased in the effluent (Figure 7.5). This coincidence also affected the SVI_5, as well. Upon the recovery of nitritation activity in continuous mode operation, the VSS_R began to increase and attained at 2.5 g VSS L^{-1}. This is a clear indication of AOB growth since only ammonium was fed into the reactor during this period. When the influent containing *o*-cresol was reintroduced, the VSS_R was kept stable, but a noticeable increase on biomass wash-out in the effluent was observed. This is mainly linked to the growth of heterotrophic bacteria able to degrade *o*-cresol. Additionally, biomass detachment and/or growth of heterotrophs partly occurred on the cell in suspension most probably contributed to the increase of VSS_E. Moreover, the increase of SVI_5 correlates with VSS_R. Nevertheless, the SVI_5 was always kept below 20 mg g^{-1} TSS which indicated that the granular biomass exhibits an excellent settleability property. The recovered granules have also shown a positive re-granulation phase during the recovery of nitritation and *o*-cresol degradation. This phase is illustrated in Table 7.2, where an increase of EPS content in the granules specifically in the polysaccharides component was observed. These results corroborate with the results obtained in Chapter 6, in which the EPS content was mainly dominated by polysaccharides than by proteins in the aerobic granular biomass treating simultaneously partial nitritation and *o*-cresol removal.

Table 7.2 Changes in extracellular polymeric substances (EPS) content of aerobic granular sludge during SAP *p*-nitrophenol (PNP), SAP phenol and SAP 2-chlorophenol (2CP) scenario events.

Period (day)	Polysaccharides (PS) (mg g VSS^{-1})	Protein (PN) (mg g VSS^{-1})	EPS (PS + PN) (mg g VSS^{-1})	Period
0	21.4 ± 1.9	9.1 ± 0.4	30.5 ± 2.3	
9	25.9 ± 1.4	12.1 ± 3.4	37.9 ± 4.8	SAP PNP
32	34.5 ± 3.0	17.7 ± 1.4	52.2 ± 4.4	
52	10.0 ± 0.5	19.1 ± 2.2	29.1 ± 2.7	SAP Phenol
71	11.7 ± 4.8	14.1 ± 1.3	25.8 ± 6.1	
90	7.5 ± 1.3	9.1 ± 0.7	16.6 ± 2.0	
103	20.9 ± 3.2	33.5 ± 2.6	54.5 ± 5.8	SAP 2CP
155	186.3 ± 26.7	10.7 ± 1.6	197.1 ± 28.3	

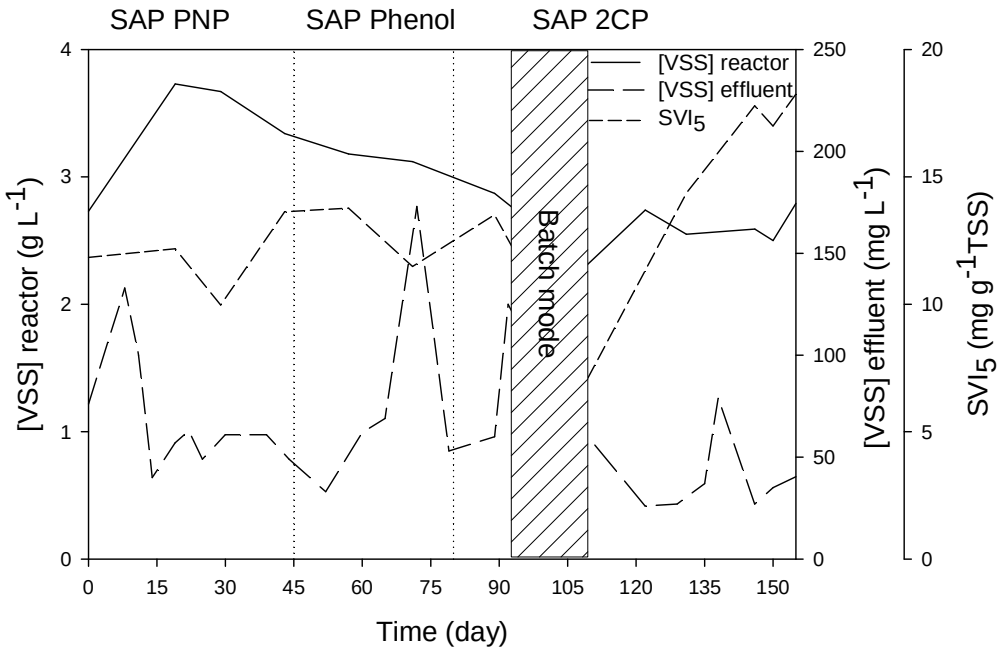

Figure 7.5 The volatile suspended solid (VSS) concentration in the reactor and in the effluent and the sludge volumetric index (SVI_5) during SAP *p*-nitrophenol (PNP), SAP phenol and SAP 2-chlorophenol (2CP) scenario events.

7.3.4 Comparison of the reactor's performance: Pre and post SAP scenarios.

A comparison on the overall performance of nitritation, *o*-cresol removal and biomass characteristics were evaluated to distinguish the general effect of SAP scenarios on the abovementioned parameters. Period I, before any SAP events began and Period XII, after three SAP events took place were selected and compared as the basis of overall reactor performance. The summary performance of the reactor in relation to both periods is tabulated in Table 7.3. The results clearly indicate that insignificant changed on the reactor performance in term of simultaneous nitritation and *o*-cresol biodegradation and biomass characteristics before and after three alternate SAP events. Although the granule size increased and NLR_v decreased in Period XII, but the NLR_S and CLR_S were almost identical when compared with Period I. The small decrease of NLR_v was strongly related to the VSS_R. On the regard of the biomass characteristics, several factors could be responsible to the changed in granule size, biomass density and settling velocity, such as influent compositions during the SAP events and operating conditions of the reactor. Certainly, the granular biomass had experienced several evolutions during the SAP events prior to Period XII. Nevertheless, the minor changed of biomass characteristics in Period XII did not affect the overall performance of the reactor. These performances were remained identical to the Period I. The findings demonstrated that the aerobic granular biomass was resilient and able to withstand the shock of toxic compounds.

Table 7.3 Summary of the performance and biomass characteristics of aerobic granular reactor before (period I) and after (period XII) sequentially alternating pollutant (SAP) events.

	Period I	**Period XII**
Granule size (mm)	1.05	1.13
Biomass density (g VSS $L_{particle}^{-1}$)	83	94
Settling velocity (m h^{-1})	42.5	49.9
Volatile suspended solid in reactor (VSS_R) (g L^{-1})	2.73	2.55
Sludge volumetric index (SVI) ratio SVI_5/SVI_{30}	1.0	1.0
Nitrite/Ammonium ratio	1.3	1.2
Volumetric nitrogen loading rate, (NLR_v) (g N $L^{-1}d^{-1}$)	1.06	0.95
Specific loading rate (nitrogen), (NLR_S) (g N VSS^{-1} $L^{-1}d^{-1}$)	0.39	0.37
Volumetric *o*-cresol loading rate, (CLR_v) (g *o*-cresol $L^{-1}d^{-1}$)	0.11	0.10
Specific loading rate (*o*-cresol), (CLR_S) (g *o*-cresol VSS^{-1} $L^{-1}d^{-1}$)	0.04	0.04
Hydraulic retention time (HRT) (d)	0.90	1.0

7.4 Conclusions

The performance of simultaneous partial nitritation and *o*-cresol removal under sequentially alternating pollutant (SAP) scenarios using an aerobic granular reactor operating in continuous mode was successfully evaluated. PNP and phenol fed (each in a separate SAP event) into the reactor were completely degraded without perturbing simultaneous partial nitritation and *o*-cresol biodegradation. However, when a secondary phenolic compound, 2CP was fed during the SAP 2CP event, partial nitritation and *o*-cresol degradation was inhibited 90 % and 25 %, respectively within three days. 2CP was exceptionally a toxic and inhibitory compound to both AOB and *o*-cresol degrading bacteria. The overall performance of the reactor in pre- and post- three alternate SAP events was remained identical. Therefore, the application of aerobic granular biomass is recommended and feasible for the treatment of complex industrial wastewaters with highly variable influent characteristics or in front of SAP events.

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Chapter 8

Simultaneous nitritation and *p*-nitrophenol (PNP) removal in a continuous airlift reactor using granular sludge

Summary

Often, chemical and petrochemical industries are producing wastewaters containing ammonium and *p*-nitrophenol (PNP). It is known that PNP has an inhibitory impact over biological processes even at low concentration, thus this compound needs to be treated accordingly. In this study, the feasibility of simultaneous nitritation and PNP biodegradation using a continuous aerobic granular reactor was evaluated. A nitrifying granular sludge developed in an airlift reactor, treating a high-strength ammonium (950 ± 25 mg $N\text{-}NH_4^+$ L^{-1}) wastewater was employed. Prior to the simultaneous biodegradation study, the airlift reactor was bioaugmented with a PNP-degrading activated sludge and immediately, PNP was added progressively in the feeding. The results indicated that nitritation was sustained during the whole of the operation period with a ca. 85 % of TAN oxidation and < 0.3 % nitrate in the effluent. However, in the first 175 days, PNP biodegradation was unstable and several accumulation episodes occurred. The oxygen limiting condition was found to be the main explanation of these events. An increase of dissolved oxygen concentration (DO) in the reactor from 2 to 4 mg O_2 L^{-1} significantly enhanced the PNP removal, achieving total elimination from day 190 onwards. At the end of experimental period, the achievable volumetric nitrogen loading rate (NLR_v) and volumetric PNP loading rate ($PNP\text{-}LR_v$) were ca. 1.0 g N $L^{-1}d^{-1}$ and 16 mg PNP $L^{-1}d^{-1}$, respectively. Analysis of fluorescent in-situ hybridization (FISH) indicated that *Acinetobacter* genus and betaproteobacterial ammonia-oxidizing bacteria were the predominant bacteria species in the granular biomass. This study has a significant implication that aerobic granular sludge could be applied potentially for developing the simultaneous removal of ammonium and phenolic compounds from industrial wastewaters.

8.1 Introduction

Several industries such as chemical, petrochemical, coke plant and refinery industry produce complex wastewaters containing both, ammonium and phenolic compounds (Morita et al., 2007; Kim et al., 2008; Milia et al., 2012). Among the numerous phenolic compounds that can be found in industrial wastewaters, *p*-nitrophenol (PNP) is one of the most phenolic compounds widely used at large scale (produced more than 1000 ton/year at least one member/country, being included in the list of High Volume Production Chemicals by Organization for Economic Cooperation and Development (OECD, 2008). Removal of PNP in wastewater can be achieved either by physico-chemical or biological treatment processes. The biological treatment demonstrates several advantages over physico-chemical processes mainly in attaining complete organic pollutants biodegradation, as well as, a low investment and operation cost (Martín-Hernández et al., 2012).

Biological treatment of wastewaters containing ammonium and PNP could be performed using activated sludge or aerobic granular sludge systems. Compared to activated sludge, aerobic granules are resistant to inhibitory and toxic compounds (Maszenan et al., 2011). Recently, reviews on the potential applications of aerobic granular sludge to treat a wide spectrum of industrial wastewaters including chemical industries influent and wastewaters containing recalcitrant organic compounds and metals have showing an interest among researchers engaging work in the area of biological wastewater treatment (Adav et al., 2009; Maszenan et al., 2011). In addition, the granules also have good settling ability, compact and strong microbial structure, and high biomass retention subsequently, increase organic degradation capacities (Adav et al., 2008). A diverse microbial community could be present in an aggregate of aerobic granular sludge unlocking the possibility to treat organic toxic or inhibitory compounds (Maszenan et al., 2011).

Several studies have been demonstrated the feasibility of aerobic granular sludge treating PNP (Yi et al., 2006; Suja et al., 2012; Yarlagadda et al., 2012) and ammonium (Bartrolí et al., 2010, 2011; Okabe et al., 2011; Yamamoto et al., 2011, among others). Many studies have shown the possibility of aerobic granules to perform simultaneous removal of ammonoium and organic matter co-existing within the same biomass aggregate (Liu and Tay, 2004; Gao et al., 2011). Although, several studies have demonstrated nitrifiers existing within the granule are sensitive and could be inhibited by the presence of phenolic compounds (Hu et al., 2005; Liu et al., 2005a; Morita et al., 2007), few studies confirmed the feasibility of simultaneous removal of both compounds (Liu et al., 2011; Suja et al., 2012). However, research on the application of simultaneous removal of ammonium and phenolic compounds using aerobic granules is still scarce and needs more attention.

A biological removal of ammonium via nitrite route by means of nitritation-denitrification or nitritation-Anammox is nowadays regarded as one of the lowest foot-print technologies for dealing with ammonium-rich wastewaters (Ahn, 2006). In view of this fact, the possibility of coupling nitritation and PNP removal using aerobic granular sludge could be considered as an attractive technology. If the proposed simultaneous biological treatment was succeed, a novel compact system capable removing both ammonium and phenolic compounds could be developed. To the best of our knowledge,

it has not been described in literature the simultaneous treatment of complex industrial wastewaters containing both ammonium and PNP by coupling nitritation and PNP removal using aerobic granular sludge.

Therefore, in this paper, the simultaneous nitritation and PNP removal using an aerobic granular sludge reactor operating in continuous mode was investigated. The performance of the granular sludge reactor treating a high- strength ammonium wastewater containing PNP was monitored and evaluated. In addition, the granular biomass was characterized throughout the experimental period, and fluorescence in-situ hybridization (FISH) technique coupled with confocal laser scanning microscopy (CLSM) technique was employed to identify the bacteria species predominant in the granules. Finally, kinetic parameters i) PNP inhibition coefficient over ammonia oxiding bacteria (AOB) and ii) oxygen affinity coefficient for PNP-degrading bacteria were also determined in this study.

8.2 Materials and Methods

8.2.1 Experimental set-up

A glass airlift reactor with a working volume of 2.6 L was utilised 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. Figure 8.1 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). 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). 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.

8.2.2 Reactor conditions and inoculums

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 continuously fed using a synthetic wastewater (see Section 8.2.3) aiming to obtain partial nitrification. Prior to the simultaneous nitritation and PNP biodegradation study, the reactor was performing partial nitritation with average effluent concentrations of: total ammonia nitrogen (TAN), 505 ± 40 mg N L^{-1}; total nitrite nitrogen (TNN), 462 ± 40 mg N L^{-1}); nitrate, 2 ± 1 mg N L^{-1} and volumetric nitrogen loading rate (NLR_v) of 0.3 ± 0.1 g N $L^{-1}d^{-1}$. The operating conditions of the reactor in the present study and the main characteristics of the granular biomass at day-0 are detailed in Table 8.1. In addition, the pH of the reactor was maintained by a regular addition of $NaHCO_3$ into the reactor.

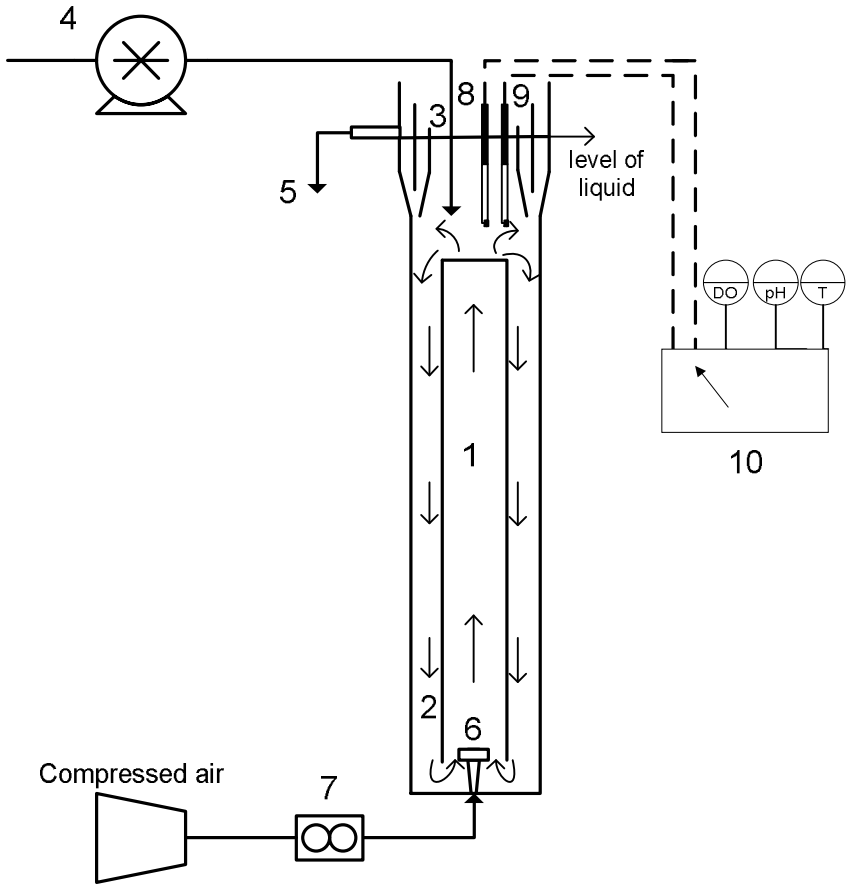

Figure 8.1 Experimental set-up of the continuous granular airlift reactor. (1) riser; (2) down comer; (3) separator; (4) feed pump; (5) effluent port; (6) air sparger; (7) rotameter; (8) pH probe; (9) DO probe; (10) monitoring panel.

Table 8.1 The operating conditions of the reactor during the study period and the main characteristics of the granular biomass at day-0.

Reactor conditions	
Temperature (ºC)	30 ± 1
Dissolved oxygen (mg O_2 L^{-1})	(1.0 to 4.5) ± 0.5
pH	8.1 ± 0.4
Hydraulic retention time (HRT) (d)	1.0 to 2.1
Air flow rate (mL min^{-1})	(125 to 500) ± 40
Biomass characteristics	
Mean size (mm)	1.1 ± 0.7
Density (gVSS $L_{particle}^{-1}$)	370 ± 140
Settling velocity (m h^{-1})	66 ± 27
Volatile suspended solid in reactor (VSS) (g L^{-1})	2.5
Sludge volumetric index (SVI_5) (mL g^{-1} TSS)	8.4
Ratio SVI_5/SVI_{30}	1.0

8.2.3 Wastewater composition

The airlift reactor was fed with synthetic wastewater with 3.63 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.

8.2.4 Bioaugmentation and the operational strategy

At day-0, the airlift reactor was bioaugmented with 500 mL (2 g VSS L^{-1}) of activated sludge from a sequencing batch reactor performing stable (PNP) degradation (Martín-Hernández et al., 2009). The bioaugmented biomass was accounted around 15 % of the total VSS inside the reactor. This percentage was selected based on the suggestion of Martín-Hernández et al. (2012) to use minimum 5 % w/w of bioaugmented biomass for ensuring the biomass is retained in the reactor for a long period. Considering that our reactor was operating in continuous mode and biomass wash out could be obvious, an excess of bioaugmented biomass was added.

At day-0, PNP was added to the synthetic wastewater and this wastewater was fed into the reactor. PNP concentrations in the influent were progressively increased during the study period (Table 8.2).

Table 8.2 PNP concentrations in the influent fed during the continuous operation of aerobic granular reactor.

Period (days)	*p*-nitrophenol concentration in influent (mg L^{-1})
0-14	5
14-119	10
120-230	15

8.2.5 Analytical methods

PNP was determined by High Performance Liquid Chromatography (HPLC) as detailed elsewhere (Martín-Hernández et al., 2009). The ammonium concentration measured as total ammonia nitrogen (TAN), the nitrite as total nitrite nitrogen (TNN) and nitrate concentrations were measured as detailed elsewhere (Bartrolí et al., 2010; Isanta et al., 2012). 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 term of size, granule density and settling velocity. 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 extracellular polymeric substances (EPS) were extracted from the granules using formaldehyde + NaOH according to Adav and Lee, (2011). The extracted sample was further analysed for EPS in term of polysaccharide and protein contents. The polysaccharide was determined using a colorimetric method with glucose as standard (Dubois et al., 1956). The protein content in the extracted sample was measured using the Lowry method with bovine serum albumin as standard (Lowry et al., 1951).

The main chemicals used in this study, *p*-Nitrophenol (PNP) (granular solid form, purity 99%) and Ammonium chloride (purity 99.5%) was 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.

8.2.5 Determination of the PNP-degraders half saturation coefficient for oxygen, $K_{O_{PNP}}$ and the PNP inhibition coefficient over AOB, $K_{I_{PNP,AOB}}$

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 DO drop 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 in the liquid phase sharply decreased because of the oxygen consumption being linked to the substrate consumption. An open and aerated LFS respirometer was employed. The respiration vessel's volume was 0.8 L and it was magnetically stirred. pH, DO 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 25 °C. The pH was maintained at 8.0 with controlled addition of HCl (1M) and NaOH (1M). The activated sludge of PNP degraders was employed from a sequencing batch reactor performing stable *p*-nitrophenol (PNP) degradation (Martín-Hernández et al., 2009). The detailed description of the $K_{O_{PNP}}$ determination and the DO profiles obtained can be found in Appendix B.

The $K_{I_{PNP,AOB}}$ determination was following the procedure proposed by Guisasola et al. (2005) and Suarez-Ojeda et al. (2007). In brief, the percentage of inhibition was determined comparing the maximum oxygen uptake rate (OUR) values measured in the control pulses before and after the inhibitor pulse. Sets of respirometric tests were performed devoted to measuring the inhibitory coefficient of PNP over nitritation. An open and aerated LFS respirometer was employed. All of the experiment set-ups were similar as previously described in the $K_{O_{PNP}}$ determination. The temperature was

maintained at 25 °C and the pH was maintained at 8 with controlled addition of HCl (1M) and NaOH (1M). The detailed description on the methodology and the typical data obtained can be found in the Appendix B.

8.2.6 FISH analysis

The fluorescence in-situ hybridization (FISH) technique coupled with confocal laser scanning microscopy (CLSM) was used to determine the fractions of the PNP oxidizing bacteria (PNP-OB), the ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the granules. 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. Hybridizations were carried out using probes targeting specific microorganisms for PNP-OB, betaproteobacteria AOB (β-AOB) and NOB (Table 8.3). The general probe consisting of equal parts of UNIV1390 and EUBmix was used for detection of all bacteria in the granules. FISH protocol for PNP-OB determination was following the procedures highlighted by Suárez-Ojeda et al. (2011). The probes used for PNP-OB were selected taking into account the characterisation previously done by these authors of a PNP-degrading biomass used in this study for developing granules with simultaneous capabilities for nitritation and PNP removal. The quantification of the microbial populations was performed following a modification of the procedure described in (Jubany et al., 2009). Prior to the quantification, the granular biomass was crushed using a mortar and a pestle and then typical FISH procedures highlighted by Suárez-Ojeda et al. (2011) was followed. To apply the quantification methodology to granular biomass (crushed), 40-50 microscopic fields were analyzed, and a single z-position was selected based on the highest intensity for each granule sample.

In order to determine the stratification of bacteria in the granules, some aggregates were cut in slices. 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 slices. Hybridizations were performed with the protocol abovementioned and the probes targeting specific microorganisms as described in Table 8.3 were used. In order to obtain a better granule staining, the amount of probe used in the hybridizations step was augmented 4 to 5 times depending on the total area of sliced granule.

Table 8.3 Probes targeting specific microorganisms employed in the FISH analysis

Probe name	Specificity	Reference
Nso190	β-AOB	Mobarry et al. (1996)
NIT3	*Nitrobacter* sp.	Wagner et al. (1996)
KO 02	*Arthrobacter* sp.	Franke-Whittle et al. (2005)
ACA652	Genus *Acinetobacter*	Wagner et al. (1994)
UNIV1390	All organisms	Zheng et al. (1996)
EUBmix	Most bacteria, planctomycetales and verrucomicrobiales	Daims et al. (1999)

8.3 Results and discussion

8.3.1 Performance of granular airlift reactor for simultaneous removal of ammonium and PNP

The performance of nitritation and PNP biodegradation in a continuous aerobic granular sludge reactor is shown in Figure 8.2 (A)-(C). At the beginning of the experimental period, the reactor was performing nitritation with an average of 62 % of TAN oxidation into TNN and < 0.3 % of nitrate in the effluent (Figure 8.2B). This nitritation performance was stably maintained until day-54 with an average NLR_v of 0.4 ± 0.1 g N $L^{-1}d^{-1}$ despite the simultaneous PNP biodegradation (Figure 8.2A and 8.2C). On day 55 until 60, a decreased in the oxidation of TAN to 27 % was recorded. Since no change has been made on the operation of the reactor, except an increase of PNP concentration in the influent from 5 to 10 mg L^{-1} on day-15, we hypothezise with two possible reasons; i) AOB activity is limiting by dissolved oxygen; ii) inhibition of AOB population by PNP. The former reason was accessed by adjusting slightly the DO in the bulk liquid to attain around 2 ± 0.5 mg O_2 L^{-1}. Immediately, the day after, the nitritation was improved and maintained for almost 120 days with an average of [TAN] of 147 ± 110 mg N L^{-1}, [TNN] of 824 ± 120 mg N L^{-1} and nitrate of 1.2 ± 0.5 mg N L^{-1} in the effluent and also, the NLR_v was maintained at around 0.42 g N $L^{-1}d^{-1}$ (Figure 8.2A and 8.2B). Again, on day-185 onward, the DO in bulk liquid was augmented to stand around 4.5 ± 0.5 mg O_2 $L^{-1}$and the NLR_v increased up to a maximum value of 1.0 g N $L^{-1}d^{-1}$ maintaining almost a full nitritation at the end of the experimental period. This value of NLR_v is comparable to those reported in the literature for conventional high-strength ammonium wastewater (i.e. 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 $L^{-1}d^{-1}$). The biomass concentration in the reactor (VSS_R) was maintained between 2.4 to 3.2 g L^{-1}, despite the changed of DO in the bulk liquid (Figure 8.4A). On day-50 until the end of the experimental period, the VSS_E and SVI_5 were always maintained at low value at around 39 ± 20 mg L^{-1} and 9 ± 2 mL g^{-1} TSS (Figure 8.4A) showing the stability of the granular sludge in the reactor during the simultaneous nitritation and PNP biodegradation.

In the present study, nitritation was always maintained with very low nitrate concentration (< 0.2 %) in the effluent despite simultaneous PNP biodegradation. From the beginning of the experimental period, a [DO]/[TAN] ratio in the bulk liquid below 0.25 was maintained to ensure the nitritation remained pronounce. The increase of DO by keeping [DO]/[TAN] ratio below 0.25, permitting to increase the NLR_v without affecting nitritation (Figure 8.2A and 8.2B, day 185 onward). This [DO]/[TAN] ratio was selected based on the results presented by Bartrolí et al. (2010) and Jemaat et al. (2013). Our results corroborate what both studies have demonstrated: by keeping an interval of ratios of [DO]/[TAN], nitrite oxidation was prevented due to the strong oxygen limiting conditions imposed in the reactor, hence allowing for obtaining and maintaining of full nitritation in continuous granular reactors. Although the DO concentration in the reactor was not too low (2 to 4.5 mg O_2 L^{-1}) the ammonium concentration was always kept in great excess. Therefore the [DO]/[TAN] concentration ratio in the reactor was very low (between 0.002 and 0.03 mg O_2 mg^{-1} TAN), outcompeting NOB in the granular sludge, as already demonstrated in previous studies (Bartrolí et al., 2010; Jemaat et al., 2013).

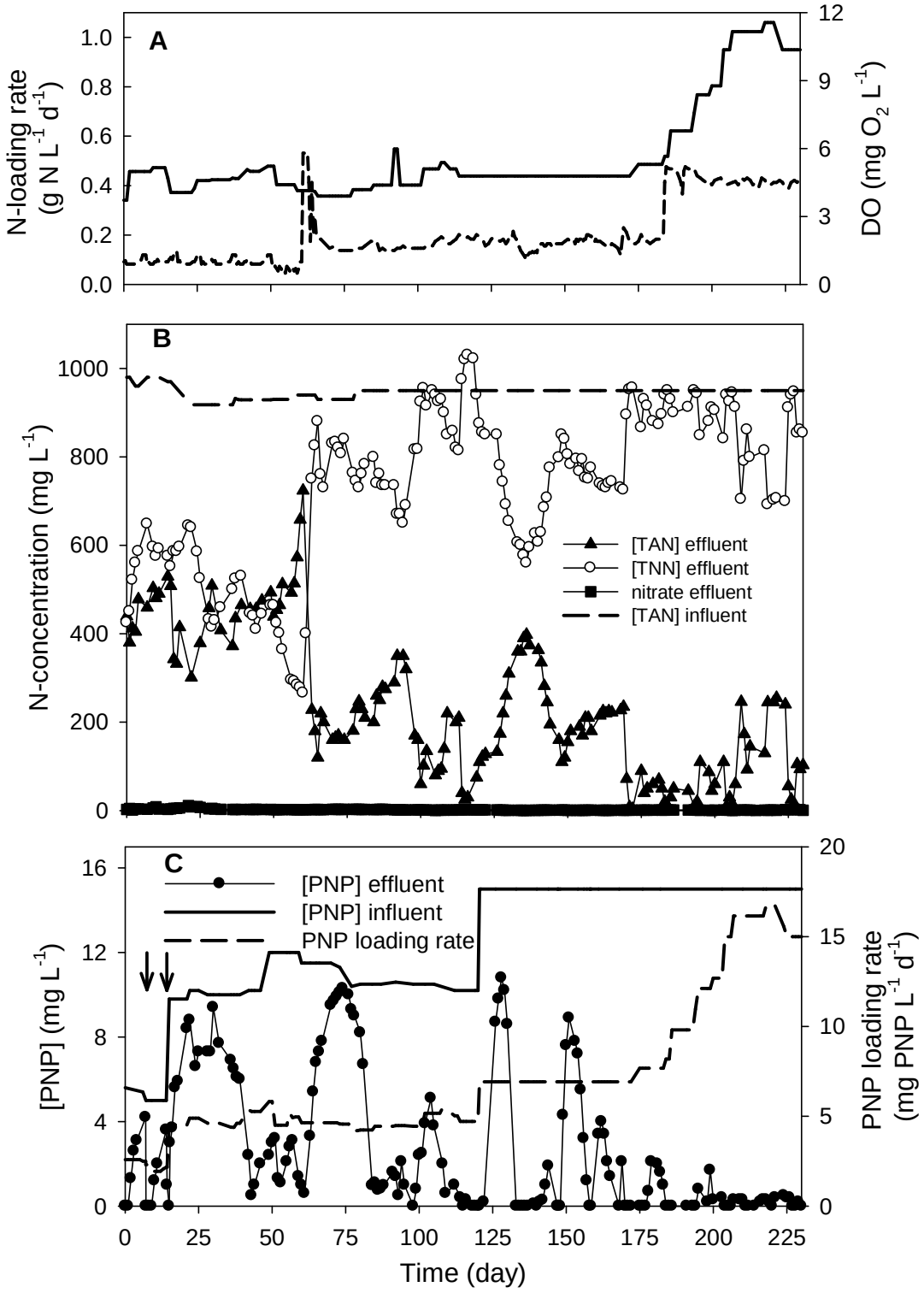

Figure 8.2 Performance of a granular nitrifying reactor treating a high-strength ammonium wastewater containing *p*-nitrophenol. (A) Volumetric nitrogen loading rate (NLR_v) and DO profile; (B) Nitritation performance; (C) *p*-nitrophenol biodegradation during the experimental period, arrows indicate 2nd and 3rd bioaugmentation events took placed on day-5 and day-10, respectively.

The hypothezise regarding the inhibition of AOB population by PNP was further investigated and a respirometric experiment was carried out. The aims of the respirometric test was to evaluate the inhibitory effect of PNP over nitritation, and to determine the PNP inhibition coefficient over AOB, $K_{I_{PNP,AOB}}$. From the respirometric experiment conducted to determine $K_{I_{PNP,AOB}}$ value, the estimated value of 7 ± 2 mg PNP L^{-1} was obtained and it's clearly indicated that the presence of PNP at around 9 mg PNP L^{-1} in wastewaters will inhibiting 50 % of AOB activity. However, the results observed in Figure 8.2 shows no clear indication of the inhibitory impact of PNP over AOB since the cyclic accumulation of PNP sometime reached 10 mg PNP L^{-1} and AOB activity did not inhibited (see for instance day-75 in Figure 8.2C). The reason that could explain this phenomenon is that granular sludge are capable to buffer the inhibitory effect better than the activated sludge (Maszenan et al., 2011). In the respirometric experiment, the $K_{I_{PNP,AOB}}$ value was determined for activated sludge, thus the real $K_{I_{PNP,AOB}}$ value for granular sludge is expected to be higher than the determined one. The observation of higher kinetic parameters for granular sludge than activated sludge was also reported in Yi et al. (2006) who determined the substrate inhibition coefficient, K_i (89.7 mg L^{-1}) for PNP by using aerobic granular sludge and found the determined K_i was significantly higher than the other systems (12-31 mg L^{-1}).

Regarding the PNP biodegradation, the percentage of PNP removal was very unstable (Figure 8.2C). In the first 5 days, the PNP accumulation was observed and this result was unexpected because from the very beginning before PNP was fed, about 15 % w/w of PNP-degrading biomass was bioaugmented into the reactor. According to Martín-Hernández et al. (2012), a minimum dosage of 5 % w/w of bioaugmented biomass is needed for implementing a successful bioaugmentation strategy. Even more, in Chapter 6, we observed a complete *o*-cresol biodegradation from the very beginning when *o*-cresol was fed into the reactor. The dosage of bioaugmented PNP-degrading biomass (12 % w/w) was almost similar to the present study. One possibility to explain the instability of PNP removal was the continuous wash-out of the specific PNP-degraders bioaugmented on day 0. Martín-Hernández et al. (2012) reported that the PNP-degrading biomass was formed by several microbial species and capable to consume other phenolic compounds as the sole carbon source. In Chapter 6, the specific microbial species able to degrade *o*-cresol was exhibited a high propensity to attach to a granule surface and thus, prevented them from being wash-out from the reactor. In the present study, an opposite behaviour of specific microbial species able to consume PNP could be plausible. For this reason, i.e. to prevent this occurence, other bioaugmentation events were carried out on days 5 and 10. However, the PNP accumulation occurred cyclically and then, it disappeared (Figure 8.2C, see 185 days downward). Further investigation was carried out on the granules morphology to examine any sign of specialised biomass developed and attached over the nitrifying granular surfaces as was expected. A closer look through the granules morphology was performed on day 1, 112 and 220 (Figure 8.3). On the first day, it was clearly seen that the nitrifying granules possed a smooth surface and regular granular shape. After 112 days and 220 days operation, it was clearly seen that the outer surface of nitrifying granules were surrounded by filamentous-liked heterotrophic bacteria that could be linked to PNP-degraders (Figure 8.3B and 8.3C). On day-220, the filamentous heterotrophic bacteria were more apparent (Fig. 8.3C). FISH analyses carried out at the end of the experimental period on the sliced granule further confirmed the existence of

heterotrophic bacteria able to degrade PNP located at the outmost layer of granule surface (Figure 8.7). This observation suggests that the PNP accumulation occurrences did not corroborate to the poor development and attachment of specialised biomass able to degrade PNP onto the nitrifying surfaces. The bioaugmentation strategy employed at the beginning of the experimental period was enhanced the formation of a diversified microbial consortium over the granules. This strategy also might help to the faster development of aerobic granules to have a better ability to withstand toxic compounds than suspended sludge.

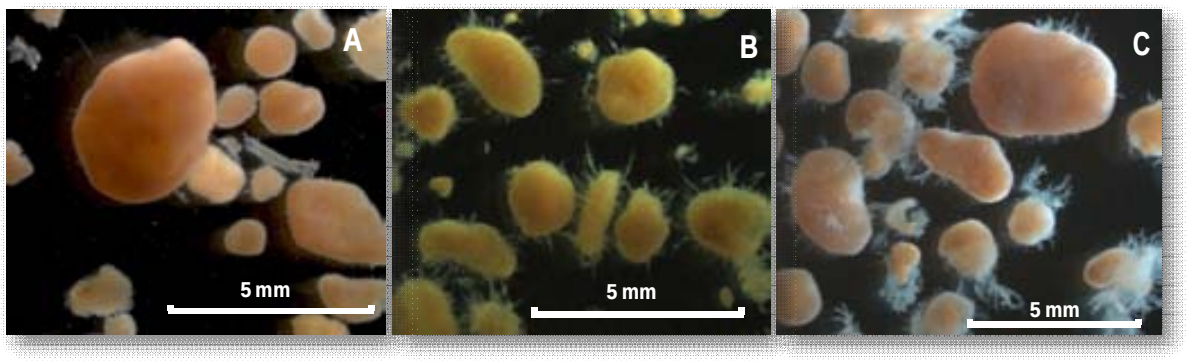

Figure 8.3 The morphology changed of aerobic granular sludge during the simultaneous nitritation and *p*-nitrophenol biodegradation. A) day-1; B) day-112; C) day-220. Scale bar = 5 mm.

Another possible reason behind the cyclically PNP coincidence could be due to the PNP biodegradation is limiting by DO. To confirm this fact, the DO concentration in the reactor was increased from 2 to 4 mg O_2 L^{-1} on day-185. From that moment and onwards, the reactor was able to remove completely all the PNP entering with the influent despite of the significant increase of the volumetric PNP loading rate (PNP-LR_v). Prior to the increase of DO, during 185 days of operation, the average PNP-LR_v was around 5 ± 2 mg PNP $L^{-1}d^{-1}$. After two fold increase of DO, the achievable PNP-LR_v was around 16 ± 1 mg PNP $L^{-1}d^{-1}$. The significant improvement on the PNP biodegradation and achievable PNP-LR_v suggest that DO concentration is the key feature in enhancing PNP biodegradation in this biofilm reactor. Similar finding was also observed by Salehi et al. (2011) who found that the oxygen supply significantly affects the PNP aerobic biodegradation. However, they determined the required DO by selecting the aeration rate instead of the DO concentration for simplification in their simulation study. Thus, it is also important to estimate the DO concentration required in order to avoid oxygen limiting condition by determining the half saturation coefficient for oxygen of PNP-degraders ($K_{O_{PNP}}$). From the respirometric experiment conducted to determine the $K_{O_{PNP}}$, the value of 1.65 ± 0.01 mg O_2 L^{-1} was calculated (Appendix B). Our estimated $K_{O_{PNP}}$ is closed to the study reported by Contreras et al. (2008), who determined the half saturation coefficient for oxygen of phenol biodegradation by activated sludge, obtained a $K_{O_{Phenol}}$ value of 1.84 mg O_2 L^{-1}. As an indication, the estimated $K_{O_{PNP}}$ value obtained in this study is relatively high compared to half saturation coefficient for AOB of 0.74 mg O_2 L^{-1} (Guisasola et al., 2005) or ordinary heterotrophic bacteria of 0.2 mg O_2 L^{-1} (Henze et al., 2000). This finding is clearly confirming our hypothesis about oxygen limiting condition for explaining the instability

of PNP removal performance in the reactor. The supplied DO concentration at 2 mg O_2 L^{-1} in the first 185 days operation period was insufficient to maintain a stable PNP degradation. Even more, a complete and stable PNP degradation was achieved after the DO concentration was augmented at 4 mg O_2 L^{-1} (Figure 8.2C). The finding suggests that the DO concentration in the bulk liquid is one of the key factors for obtaining a complete and stable PNP removal in a granular sludge reactor.

8.3.2 Biomass characterization

Figure 8.4B illustrates the granular characteristics were significantly affected during the simultaneous nitritation and PNP biodegradation. The granule size was increased from 1.1 mm to 1.6 mm at the end of the operation period. It was also recorded that during the first 100 days, the granule size slightly decreased possibly due to the increased of shear force caused by the increased of DO from 1 to 2 mg O_2 L^{-1}. Again, on day-185 onward, the DO was augmented to attain at 4 mg O_2 L^{-1}. During this period, the reactor was able to treat higher loading rate for both ammonium and PNP than before. The changed of these two factors, especially on the loading rate certainly had promoted the growth of bacteria, thus increased the size of granule, observed at the end of the experimental period..

The biomass density was decreased at the end of the reactor operation (Figure 8.4B). The bioaugmentation strategy employed at day-zero had enhanced the formation of a diversified microbial consortium over the granules. The development and attachment of heterotrophic biomass able to degrade PNP onto the nitrifying surfaces (Figure 8.7) had an impact on the structure of granules. The heterotrophs is thought to produce much more polysaccharides than nitrifying bacteria (Yang et al., 2005). It seems reasonable to consider that the production of polysaccharides would increase the degree of separation between the cell cluster and this in turn reduces the strength of the granule structure i.e. biomass density.

It was observed that the change of settling velocity of granules during the experimental period followed the similar trend with the size and density of the granules (Fig. 8.4B). For instance, the decrease of settling velocity during 100 days of operation is due to the decrease in size and density of the granules. Similar trend of settling velocity was also observed after 100 days of operation period. The correlation between settling velocity and the granule size found in this study was corroborated with a generalised model for aerobic granular sludge proposed by (Liu et al., 2005b).

Table 8.3 shows the EPS changes in the granular sludge during the simultaneous nitritation and PNP biodegradation. The EPS content was a measured of the total concentrations of polysaccharides (PS) and protein (PN). The results indicated that during 178 days of operation, the EPS was unchanged. However, at the end of the reactor operation, the EPS content was increased. An increased of EPS at this period is strongly linked to the operation of the reactor. Two operational changed were imposed; i) increased in DO (shear force increased) and ii) increased in loading rate. Both actions had stimulated the microbial activity and thus increased the production of EPS. The stimulated microbial activity and production of EPS was also observed by Tay et al., (2001a). In general, higher concentration of PS than PN during the whole experimental period was observed (Table 8.3). Consequently, the ratios of PS/PN were always more

than 1. The high PS content in EPS was also reported in several studies of 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., 2008b). The results suggest that the granular sludge fed with the PS could be dominant compounds of EPS in the aerobic granules feeding continuously with a high-strength ammonium wastewater and PNP.

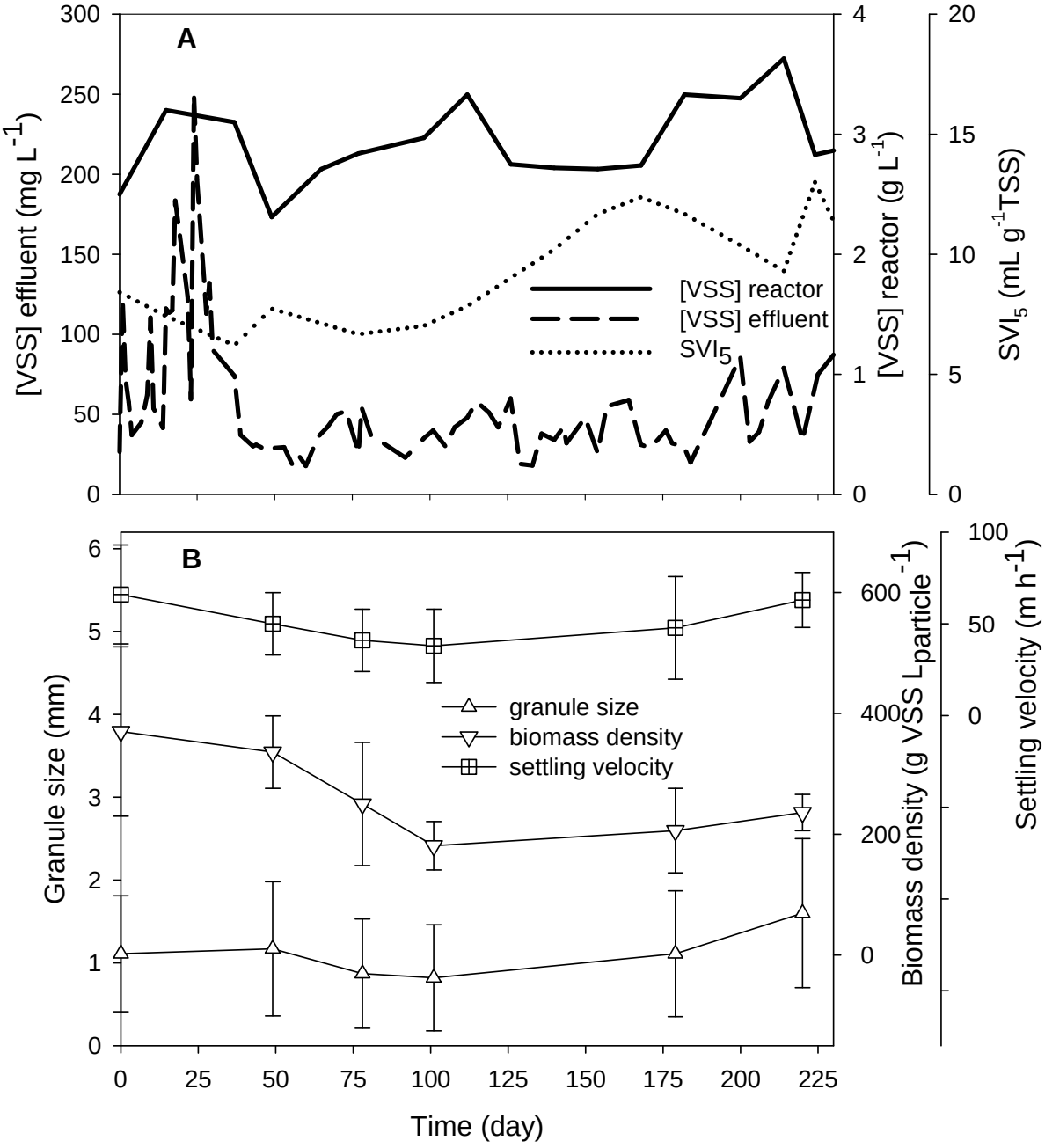

Figure 8.4 The biomass characteristics in term of; (A) volatile suspended solid (VSS) concentrations and SVI values; (B) granule size, biomass density and settling velocity in a granular reactor during the operational period.

Table 8.3 Changes in extracellular polymeric substances (EPS) content of aerobic granular sludge during the simultaneous nitritation and PNP biodegradation.

Period (day)	Polysaccharides (PS) (mg g VSS^{-1})	Protein (PN) (mg g VSS^{-1})	EPS (PS +PN) (mg g VSS^{-1})	Ratio PS/PN
0.	22 ± 2	22 ± 1	44 ± 3	1.00
49	19 ± 7	12 ± 1	31 ± 8	1.58
101	27 ± 3	14 ± 1	41 ± 4	1.92
178	31 ± 2	8 ± 2	39 ± 4	3.88
220	36 ± 6	24 ± 1	60 ± 7	1.50

8.3.3 Identification of dominant species using FISH technique

The results from the FISH technique identified that only *Acinetobacter* genus and β-AOB were the predominant populations, whereas *Nitrobacter* sp. was detected but at very low occurrence (Figure 8.5). *Arthrobacter* sp. was not detected in the sample. The images gathered were further quantified to estimate the percentage of bacteria species present in the biomass. It was estimated that about 52 ± 14 % of *Acinetobacter* genus, 49 ± 20 % of β-AOB and 1 ± 1 % of *Nitrobacter* sp. The *Acinetobacter* genus is believed to be the responsible for PNP biodegradation, meanwhile, β-AOB would be responsible of the nitritation process. *Acinetobacter* genus seems to be preferentially in the outer layer, but some are also inside the granule (Figure 8.6A). β-AOB is distributed in the whole granule but also in the outer layer (Figure 8.6B) and *Nitrobacter* sp. is almost undetectable (Figure 8.6C). The microbial distribution observed in this study is different of what we had observed in Chapter 6. Nevertheless, seems that the hypothesis of microbial stratification following different metabolic activities could be plausible.

Acinetobacter genus detected in our biomass was also observed by (Suárez-Ojeda et al., 2011) and corroborates with one of the bacteria population dominant in the bioaugmented PNP-degraders (Pramparo et al., 2012). According to these authors, in that biomass *Arthrobacter* sp. fraction accounted for 30 ± 13% of the total bacteria while genus *Acinetobacter* fraction was 31 ± 15% (Pramparo et al. 2012). Our results seems to indicate that only *Acinetobacter* genus and not *Arthrobacter* sp. was capable of being retained in the granular airlift reactor, maybe because the former have better auto-aggregation properties than the later. More specific studies are needed to confirm this extreme.

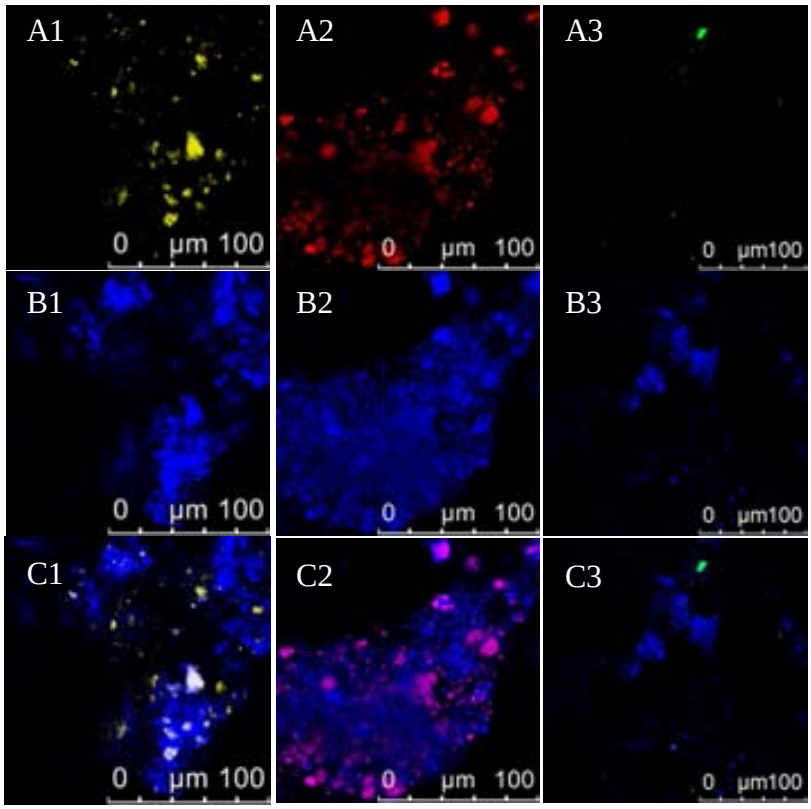

Figure 8.5 FISH image of a sample of crushed granules collected at the end of the experimental period (Bar = 100 µm). Yellow: *Acinetobacter* genus (ACA652); Red: βAOB (Nso190); Green: *Nitrobacter* sp. (NIT3); Blue: all bacteria (EUBmix+UNIV1390); First row (A) correspond to the specific probe, second row (B) to the general probe and third row (C) to the merge image.

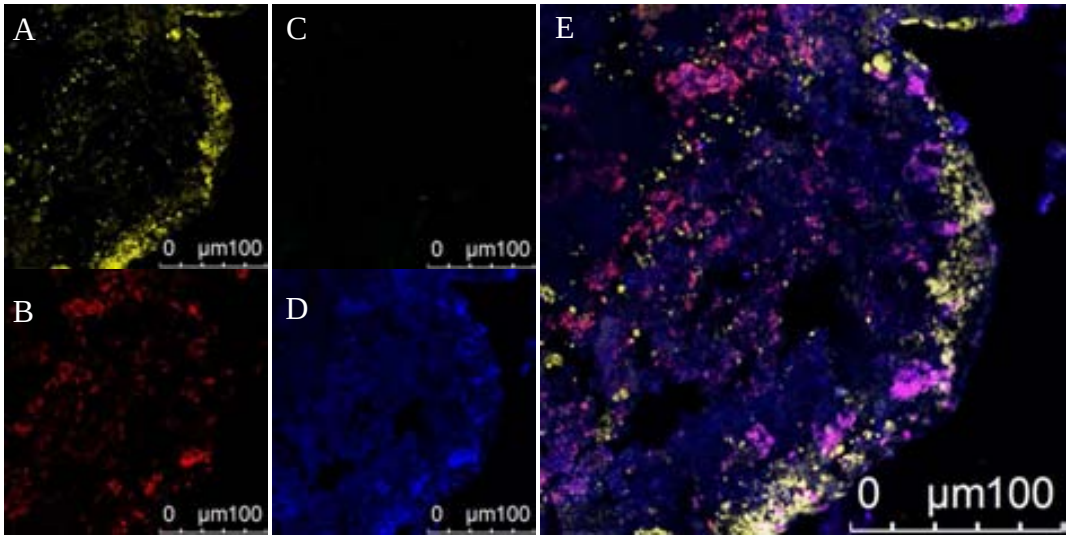

Figure 8.6 FISH image of a sliced granules collected at the end of the experimental period (Bar = 100 µm). A) Yellow: *Acinetobacter* genus (ACA652); B) Red: Betaproteobacteria AOB (Nso190); C) Green: *Nitrobacter* sp. (NIT3); D) Blue: all bacteria (EUBmix+UNIV1390); E) Merge image. Centre of the granules is on the upper left corner.

8.4 Conclusions

In this study, the simultaneous nitritation and PNP biodegradation was successfully demonstrated in a single reactor using aerobic granular sludge. Nitritation was maintained in the reactor for more than 185 days despite unstable PNP biodegradation. The oxygen limiting conditions was the key factor affecting the instability of PNP biodegradation in the continuous aerobic granular reactor. This reason was further supported by the estimation of $K_{O_{PNP}}$, value of 1.65 ± 0.01 mg O_2 L^{-1} and it confirmed that the DO concentration applied at 2 mg O_2 L^{-1} was insufficient. At DO of 4.5 mg O_2 L^{-1}, a complete degradation of PNP was achieved and stably maintained until the end of the experimental period. Concurrently, the NLR_v and PNP-LR_v also were increased and attained at around 1.1 ± 0.1 g N $L^{-1}d^{-1}$ and 16 ± 2 mg PNP $L^{-1}d^{-1}$, respectively. The finding suggests that the DO concentration should be applied appropriately to ensure a stable PNP biodegradation in aerobic treatment. In addition, the granular characteristics were significantly affected during the simultanueos nitritation and PNP biodegradation. The bioaugmentation strategy applied at day-0 was enhanced the formation of a diversified microbial consortium over the granules and it help to the faster development of aerobic granules to have a better ability to withstand toxic compounds than suspended sludge. This suggests that an aerobic granular system is a feasible application for simultaneous removal of ammonium and phenolic compounds. In the near future, we propose the simultaneous nitritation and PNP removal should be coupled with heterotrophic denitrification for sustainable nitrogen removal.

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Chapter 9

Starvation and subsequent reactivation of an aerobic granular reactor treating simultaneously ammonium and *p*-nitrophenol

Summary

Industrial wastewaters are generated according to the upstream process schedule; therefore, its composition and flow-rate can be highly variable. Often, low activity periods or total production shut down would even result in complete interruptions of wastewater inflows (starvation) to the industrial wastewater treatment plants (IWWTPs). In this case a reliable and robust biological wastewater treatment is required to cope with those difficult periods. In this study, the impact of starvation periods and the subsequent reactivation of an aerobic granular reactor performing simultaneous nitritation and *p*-nitrophenol (PNP) removal were investigated. Two starvation scenarios were applied and evaluated i) a PNP starvation period, in which PNP was omitted in the feeding during two weeks; and ii) a total starvation period by swithing off the continuous operation for two weeks. Full recovery of PNP degradation was achieved in 2 days after the PNP starvation period. Nitritation was unaffected during and after the PNP starvation period, showing the robustness of the aerobic granular system. Regarding to total starvation period, full recovery of simultaneous nitritation and PNP removal was accomplished in only 11 days. The results demonstrated that the aerobic granular biomass is reliable, robust and capable to recover its biodegradation performances during long-term starvation periods either due to the WWTPs shutdown for maintenance purposes or production variability.

9.1 Introduction

Several industry sectors such as chemical, petrochemical, coke plants and pharmaceutical are known to generate wastewaters containing ammonium and phenolic compounds (Kulkarni and Chaudhari, 2007; Morita et al., 2007; Milia et al., 2012). Currently, the treatment of these effluents is partial and costly, since the solution adopted is often a physic-chemical treatment which does not completely remove the contaminants (Oller et al., 2011). Biological treatment offers a complete biodegradation of contaminants and associates with low investment and operational costs. However, sudden changes in the inflow or variable influent characteristics are a common challenge faced by biological treatment processes when receiving industrial wastewater (Sipma et al., 2010). In particular, low activity periods or an eventual production shut down would result in a complete interruption of the wastewater inflow to the industrial wastewater treatment plants (IWWTPs) and therefore the starvation of the biological processes (Yilmaz et al., 2007; Torà et al., 2011). During that periods, the biodegradation capacity can be seriously affected (Torà et al., 2011). Therefore, it is crucial and important to maintain the viability of biomass during starvation periods as well as to uphold a fast reactivation of the biological treatment capacity when inflow conditions are restored.

Besides of smoothing operational parameters during the restart of the reactor after starvation ends, several strategies could be adopted during these difficult periods either to reliably preserve the biomass or to achieve a fast start-up of the biological treatment;

i) Storing the biomass at 4 °C or between 20 °C and 26 °C (Gao et al., 2012; Yuan et al., 2012).
ii) Settling and leaving the biomass in the reactor under anoxic conditions and at ambient temperature (Torà et al., 2011).
iii) Bioaugmentation of specific degrading bacteria (Tyagi et al., 2011). This can be done in one-off or intermittent additions.

The impact of long-term starvation periods and subsequent (fast) reactivation of the biological processes strongly depend on the decay rate of bacteria. In general, it has been found that avoiding completely the aerobic conditions resulted in the lowest bacteria decay rates; hence it has been recommended for maintaining bacteria activity in WWTPs during periods without feed (Lee and Oleszkiewicz, 2003; Yilmaz et al., 2007; Torà et al., 2011).

Biological treatment of wastewaters containing ammonium and phenolic compounds could be performed using activated sludge or aerobic granular sludge systems. Compared to activated sludge, aerobic granules are resistant to inhibitory and toxic compounds (Maszenan et al., 2011). In addition, the granules also have good settling ability, compact and strong microbial structure, and high biomass retention subsequently, the organic degradation capacities (Adav et al., 2008). The potential applications of aerobic granular sludge to treat a wide spectrum of industrial wastewaters including chemical industries influent and wastewaters containing recalcitrant organic compounds and metals is increasingly drawing interest researchers engaging work in the area of biological wastewater treatment due to the good performance and cost-effectiveness of the treatment (Adav et al., 2009;

Maszenan et al., 2011). Up to date, most of the aerobic granules starvation studies are related to the formation and granulation of granular sludge (Li et al., 2006; Wang et al., 2006; Liu and Tay, 2008; Gao et al., 2011). However, few studies have been focused in long-term starvation or storage periods and subsequent reactivation on matured aerobic granules (Zeng et al., 2007; Pijuan et al., 2009; Xu et al., 2010; Gao et al., 2012; Yuan et al., 2012). Therefore, it is crucial to investigate the ability of the granules and the biomass to survive under starvation or storage periods and subsequent reactivation of the biological capacity in order to tackle the reliability of this biological wastewater treatment. To the best of our knowledge, there are no studies on the impact of starvation periods and subsequent reactivation of an aerobic granular reactor operating in continuous mode performing simultaneous nitritation and phenolic compounds removal. The reported studies in the literature were only focusing on long-term starvation periods of aerobic granular biomass performing only nitrification, COD or phosphorus removal (Wang et al., 2008; Pijuan et al., 2009; Gao et al., 2012; Yuan et al., 2012)

In this sense, the goal of this study is to investigate the impact of long-term starvation periods on an aerobic granular reactor performing simultaneous nitritation and *p*-nitrophenol (PNP) removal in continuous mode and its subsequent reactivation. The starvation conditions under investigation were: i) impact of starvation of one substrate (PNP) ii) impact of total starvation (reactor shutdown). The behaviour of aerobic granules under those conditions was also evaluated by measuring their physical properties after each of the starvation periods.

9.2 Materials and Methods

9.2.1 Chemicals and reagents

p-Nitrophenol (PNP) in a granular solid form (purity 99%) was employed and supplied by Panreac (Spain). Ammonium chloride (purity 99.5%) used was supplied by Carl Roth (Germany). All the chemicals and other reagents were purchased from Sigma-Aldrich (Spain) and the highest purities available were employed.

9.2.2 Reactor set-up, operations and wastewater

A glass airlift reactor with a working volume of 2.6 L was used 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. Figure 6.1 depicts the detail of 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 (Crison pH 5333) probes 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). Feeding to the reactor was made with a membrane pump (ProMinent Gamma/L). DO in the reactor was regulated by a rotameter (Aalborg, USA). The detail of the experimental set-up can be found in Chapter 8.

Before the starvation study, the aerobic granular reactor was fed continuously with synthetic high-strength ammonium wastewater (950 ± 25 mg $N\text{-}NH_4^+$ L^{-1}) and PNP

(15 mg L^{-1}) over a period of 8 months (refer to Chapter 8). During this period, the reactor was performing simultaneously full nitritation and PNP removal. The operating conditions of temperature and DO were 30 ± 1.0 ºC and 4 ± 0.5 mg O_2 L^{-1}, respectively. The pH of the reactor was maintained at 8.1 ± 0.4 by a regular addition of $NaHCO_3$ in the reactor. The detailed composition of the synthetic wastewater were 3.63 g L^{-1} NH_4Cl (950 ± 25 mg N-NH_4^+ L^{-1}) and PNP (15 mg 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.

9.2.3 Starvation and reactivation experiments

The stability and subsequent reactivation of the aerobic granular reactor performing simultaneously nitritation and PNP removal were assessed in two different starvation periods.

i) PNP starvation: PNP was omitted in the influent for a period of two weeks. During the PNP starvation period, the aerobic granular reactor was operating in continuous mode treating the synthetic high-strength ammonium wastewater. The regular feeding containing ammonium and PNP was resumed after 15 days and reactivation of PNP removal and stability of nitritation and simultaneous removal of ammonium and PNP were evaluated. The operating conditions during and after the PNP starvation period i.e temperature, DO, pH were remained unchanged as described in Section 9.2.2.

ii) Total starvation: The continuous operation of aerobic granular reactor was completely stopped for a period of two weeks. The feeding, temperature regulator, and aeration of the reactor were switched off. The granules were left to settle down at the bottom of the reactor with its own supernatant. During the total starvation period, the pH was around 8.7 ± 0.2, and the temperature was 24 ± 2 °C (room temperature). Also, the DO was below 0.5 mg O_2 L^{-1}. After two weeks of total shut-down, the reactor was restarted and all the operating parameters (temperature, 30 ± 1.0 ºC; DO, 4 ± 0.5 mg O_2 L^{-1}; pH, 8.1 ± 0.4) were kept similar as described in Section 9.2.2. but continuous operation was not restarted. Instead, a pulse of ammonium was introduced into the reactor. This way of resuming the reactor operation after the total starvation period was purposely selected to assess the recovery of ammonium oxidation. Upon confirming the reactivation of ammonium oxidation, the reactor was switched to continuous operating mode, and an influent containing both ammonium and PNP was re-fed to the reactor. The inflow rate was progressively increased until attaining the previous value. The performance of simultaneous nitritation and PNP removal was evaluated accordingly.

The detail of operational strategy imposed to the aerobic granular reactor during the whole experimental period is summarized in Table 9.1.

Table 9.1 Operation mode and main influent compositions imposed to the aerobic granular reactor during the starvation periods study.

Time (days)	Operation mode	Influent compositions
0-21	Continuous	$N\text{-}NH_4^+$ + PNP
22-35	Continuous (PNP starvation)	$N\text{-}NH_4^+$
36-62	Continuous	$N\text{-}NH_4^+$ + PNP
63-77	Total starvation	no influent
78-89	Batch*	$N\text{-}NH_4^+$*
90-135	Continuous	$N\text{-}NH_4^+$ + PNP

*reactor restarted and a pulse of $N\text{-}NH_4^+$ was directly introduced into the reactor

9.2.4 Analytical methods

PNP was determined by High Performance Liquid Chromatography (HPLC) (UltiMate 3000, Dionex Corporation) using an Agilent Zorbax SB-C18 (4.6 x 100 mm, 3.5 μm) column and a UV detector set at 254 nm, the flow rate was 1.875 mL min^{-1} and the column temperature was maintained at 30 ºC (Martín-Hernández et al., 2009). The mobile phases were ultrapure water containing H_2SO_4 at pH 1.41 and HPLC grade methanol following a gradient elution. The gradient started from 100% of acidified water and progressively changed to 50:50 v/v of water:methanol in 18 min, then it remained isocratic until 20 min. The injection volume was 20 μL and the maximum pressure in the column was approximately 290 bars. The ammonium concentration measured as total ammonia nitrogen (TAN) was analyzed using a continuous flow analyzer based on potentiometric determination. The nitrite and nitrate were measured with ionic chromatography (ICS-2000 Integrated Reagent-Free IC System, DIONEX). 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 term of size, granule density and settling velocity. 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 images were further processed using Image-Pro Plus version 6.0 (Media Cybernetics, Inc.,). The detail procedures can be found in Chapter 8. The density of the granular sludge was determined using the dextran blue method described by Beun et al. (2002). The settling velocity was determined by placing individual granules in a column containing the described wastewater and measuring the time spent to drop a height of 40 cm (Bartrolí et al., 2010).

9.3 Results and discussion

9.3.1 Reactor performance before, during and after the PNP starvation period and subsequent reactivation of PNP degradation.

Before the starvation experiment, the aerobic granular reactor was stably operating. Nitritation was maintained with an average of 92 % of nitrite accumulation and 8 %

of ammonium in the effluent (Figure 9.1). Nitrate concentration was always below 1.5 ± 0.5 mg N L^{-1}. Simultaneously, a complete PNP removal was maintained at any time. The average volumetric nitrogen loading rate (NLR_v) and volumetric PNP loading rate (PNP-LR_v) were 0.95 g N $L^{-1}d^{-1}$ and 15 mg PNP $L^{-1}d^{-1}$, respectively. Additionally, the average biomass concentration in the reactor was around 2.90 g L^{-1}.

In the PNP starvation period, PNP was omitted in the feeding for a period of 14 days. During the starvation period the nitritation was stably maintained, and the oxidation of ammonium was ca. 90% (Figure 9.1.B). After the PNP starvation period, the PNP was re-introduced in the influent again with a concentration of 15 mg PNP L^{-1}. During a period of 2 days PNP built up to 12 mg L^{-1} (Figure 9.1C). Despite the presence of a relatively high PNP concentration in the reactor bulk liquid, the performance of nitritation was maintained stable during this period and the NLR_v applied was 0.95 g N $L^{-1}d^{-1}$ (Table 9.2). The inhibition of AOB by PNP was studied previously in Chapter 8, and a value of 7±2 mg PNP L^{-1} for the half saturation coefficient was obtained. Since no impact on nitritation was detected, we hypothesize with two possible reasons, which are not mutually exclusive: (i) acclimation of AOB to PNP (the reactor was treating a wastewater containing PNP for 8 months prior the starvation period); (ii) granular sludge is contributing to buffer the inhibitory effects (because half-saturation coefficient was determined for activated sludge).

The short PNP building up period was followed by a fast decrease until the complete removal was again steadily achieved (Figure 9.1C, from day 40 to 62). The full recovery of PNP removal activity is also supported by the PNP-LR_v achieved, i.e. 15 mg PNP $L^{-1}d^{-1}$ identical to that applied before the starvation period (Table 9.2). The ability of aerobic granules to sustain its PNP degradation activity after 14 days PNP starvation period and only took four days to reactivate and full recovery its degradation activity was remarkable. In reviewing the literature, the study of simultaneous nitritation and PNP removal using a continuous aerobic granular reactor under the PNP starvation period and subsequent reactivation are not previously reported. However, several studies on toxic compounds under starvation periods were reported and could be compared to our study. Osuna et al. (2008) and Emanuelsson et al. (2008) evaluated the simultaneous biodegradation of 2-flourobenzoate (2-FB) and dichloromethane (DCM) using immobilized biomass onto expended clay (EC) under 20 and 22 days substrate (2-FB) starvation periods and they reported that it took ca. 15 and 8 days, respectively for 2-FB to attain its degradation performance after re-feeding of 2-FB influent in EC reactor. A fast recovery of toxic compound (PNP) degradation (4 days) observed in the present study compared to those studies could possibly due to the buffering capacity of granular biomass to sustain substrates inside the granules. Recent research has demonstrated that granular sludge may retain by sorption low quantities of substrate which is thought to contribute to buffer the negative effects of starvation (Buitrón and Moreno-Andrade (2011)).

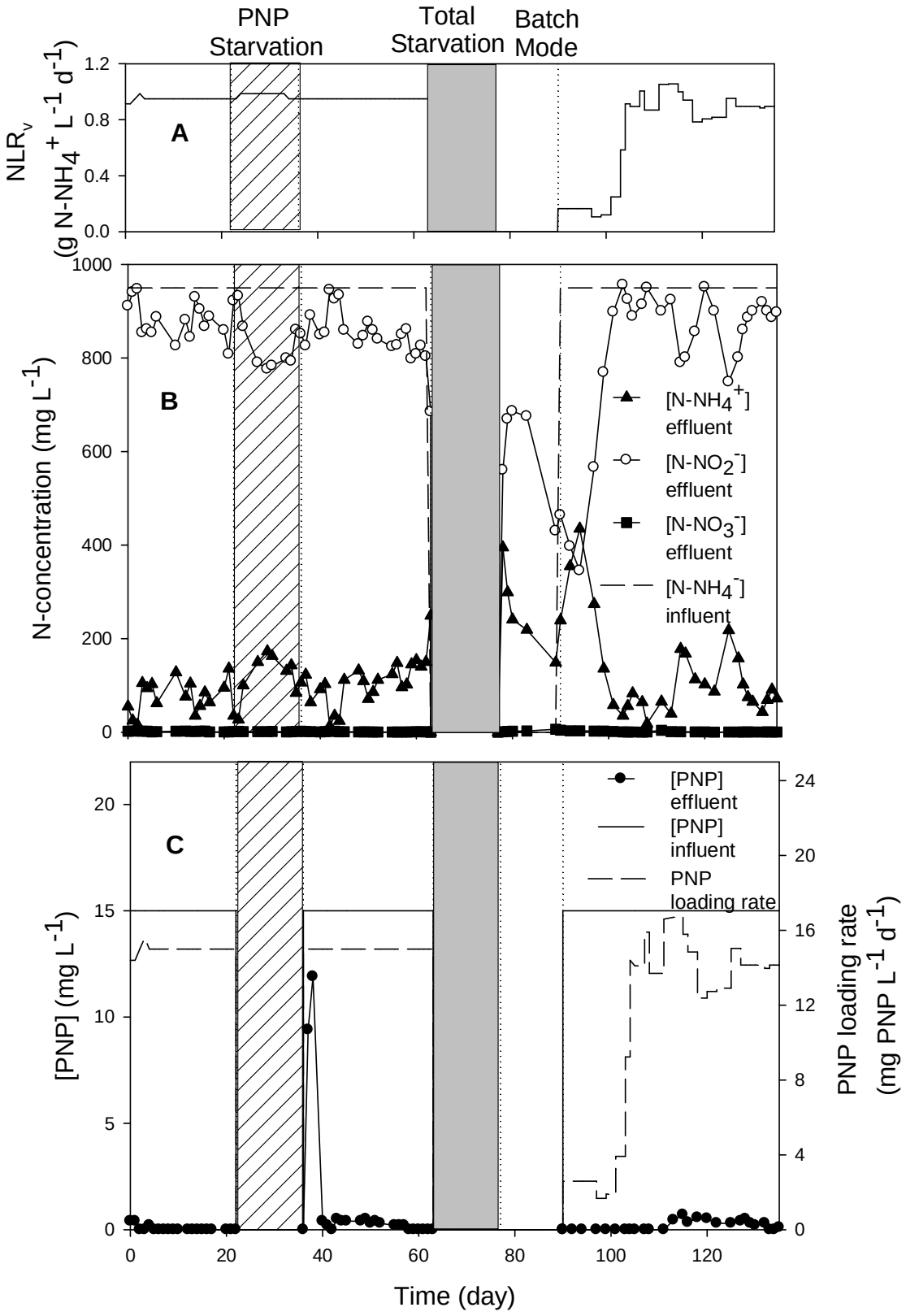

Figure 9.1 Performance of the aerobic granular reactor treating simultaneously ammonium and *p*-nitrophenol (PNP) under PNP and total starvation periods. (A) Volumetric nitrogen loading rate (NLR_v); (B) Performance of nitritation; (C) Performance of PNP removal. A pulse of N-NH_4^+ (final concentration was ca. 400 mg N L^{-1} in bulk liquid of the reactor) was directly introduced into the reactor on day 78.

Granular sludge characteristics were not importantly affected by the PNP starvation period, because size, SVI_5, SVI_5/SVI_{30} and settling velocity are inside the common ranges reported for granular sludge (Table 9.2 and Figure 9.2). A slight decrease in granule size, biomass concentration and granule density was detected (Table 9.2 and Figure 9.2), this is linked to the wash out of biomass from the reactor during the PNP starvation period, as supported by the time curse solids concentration measured in the effluent (Figure 9.2). In spite of this biomass decrease, nitritation was unaffected and PNP removal recovery after resuming the regular operation of the reactor was fast, as already discussed.

Table 9.2 Granules characteristics at different experimental days during the starvation study.

	Day 1	Day 62	Day 130
Physical properties			
Granule size (mm)	1.60 ± 0.63	1.31 ± 0.86	1.33 ± 0.81
Biomass density (g VSS $L_{particle}^{-1}$)	236 ± 22	160 ± 31	204 ± 68
Settling velocity (m h^{-1})	63 ± 14.6	48 ± 9.4	59 ± 19.4
Volatile suspended solid in reactor (VSS_R) (g L^{-1})	2.83	2.23	2.41
Volatile suspended solid in effluent (VSS_E) (mg L^{-1})	34	281	74
Sludge volumetric index (SVI_5)	13	14.1	12.7
Ratio SVI_5/SVI_{30}	1.0	1.0	1.0
Reactor performance			
Average N-NH_4^+ oxidation (%)	92.1	89.5	93.0
Average PNP removal (%)	99.6	98.3	98.3
Volumetric nitrogen loading rate, (NLR_v) (g N $L^{-1}d^{-1}$)	0.95	0.95	0.90
Specific loading rate, (NLR_S) (g N VSS^{-1} $L^{-1}d^{-1}$)	0.34	0.42	0.37
Volumetric PNP loading rate, (PNP-LR_v) (mg PNP $L^{-1}d^{-1}$)	15	15	14.2
Specific loading rate (PNP-LR_S) (mg PNP VSS^{-1} $L^{-1}d^{-1}$)	5.3	6.7	5.9
Hydraulic retention time (HRT) (d)	1.0	1.0	1.1

9.3.2 Reactor performance after the total starvation period

Upon completion of PNP starvation experiment, the reactor was left operating at steady state conditions for more than 20 days. Then, the total starvation experiment was carried out to simulate an operational shut-down period. In this experiment, the operation of the reactor was completely switched off, and the aerobic granules were left to settle down at the bottom of the reactor for 14 days. Appropriate strategies need to be implemented in order to minimize the post-impact of starvation and subsequently speed-up the reactivation and recovery of biological processes (Cabezas et al., 2009; Torà et al., 2011). In the present study, the reactor was operated in a batch mode during the start-up period is to ensure an appropriate ammonium load was

fed in the reactor (Cabezas et al., 2009). This strategy also aims to reactivate AOB activity and subsequently recover nitritation prior to reactivation of PNP degradation since nitrifiers decayed slower during starvation and reinstated rapider during reactivation than heterotrophs ((Wang et al., 2008). On the first day of the reactor start-up, a pulse of ammonium was introduced in the reactor and ammonium oxidation rate was closely monitored. The results illustrated that the reactivation of nitritation was pronounced from the first day of reactor operation (Figure 9.1B). It signifies that ammonium oxidizing bacteria (AOB) remain survived in the granular biomass under 14 days of total starvation period. Torà et al., (2011) suggested that it would be better to restart partial nitrification in a period which not exceeded 2 weeks (starvation or shutdown periods) in order to avoid a significant decrease of AOB biomass and this restart period was implemented in the present study. A longer starvation period of aerobic granules would negatively impact the recovery period of nitrifiers activities as reported by Yuan et al. (2012); 6 months storage, only 30% recovery of nitritication after 7 days, Gao et al. (2012); 8 months storage, recovered almost all nutrients removal capacity within 1 month and Pijuan et al. (2009); 4 weeks storage, full recovery of nitrification in 3 weeks.

The reactor operation was switched from batch mode to continuous mode 11 days after total starvation (Figure 9.1B). Simultaneously, the re-introduction of ammonium and PNP in the reactor was initiated. During this period, the NLR_v was progressively increased. The results showed that, during the continuous operation, it required 11 days for nitritation to recover its performance attained the previous nitrite accumulation (ca. 90 % of nitrite accumulation before the total starvation period). The nitritation performance was maintained steady during several weeks showing the stability of recovered nitritation system. Interestingly, no nitrate built-up was observed in any experimental period and no PNP accumulation, in spite of PNP-degrader being starved from PNP during 25 days. This is proved that the strategy imposed to achieve and maintain stable nitritation by keeping an appropriate ammonium load (Cabezas et al., 2009) and [DO]/[N-NH_4^+] ratio (Bartrolí et al., 2010; Jemaat et al., 2013) was successful applied for fast reactivation and recovery of nitritation. A high ammonium accumulation was observed on day 95 resulting into a slight adjustment of NLR_v. At the end of the experimental period, the NLR_v (0.90 g N $L^{-1}d^{-1}$) achieved was slightly lower than the NLR_v achieved before the total starvation period (0.95 g N $L^{-1}d^{-1}$). The strategy to keep an appropriate ammonium load during the recovery phase directly affected the PNP load imposed to the reactor. Consequently, PNP was consumed and completely removed in the effluent. It is important to note that there was no accumulation of PNP was observed from the very first day of PNP reintroduction as happened in the previous PNP starvation period study (Figure 9.1C). In both reactivation periods (after PNP starvation and total starvation periods), the only operational differences were the loading strategy imposed. In the total starvation experiment, PNP-LR_v was progressively increased, whereas, in the PNP starvation experiment, similar PNP-LR_v value before the starvation period was imposed directly to the reactor. This finding indicates that by progressively increasing the PNP loading rate, the inhibitory impact of PNP could be reduced significantly, thus a fast reactivation and recovery of PNP degradation could be accomplished. Nevertheless, the overall performance of recovered nitritation system using aerobic granular reactor was significant and almost identical to the previous state before the total starvation occurrence. The results demonstrated that the performance of simultaneous nitritation and PNP removal in an aerobic granular

reactor could be easily recovered after a shutdown of the upstream process for periods of at least 14 days. In the present study, full recovery of nitritation and PNP removal system attained in 21 days by applying a batch mode operation strategy at the beginning and then switching to a continuous operation and, keeping an appropriate substrates loading to the reactor

Granular sludge characteristics were not importantly affected by the PNP starvation period, because size, SVI_5, SVI_5/SVI_{30} and settling velocity are inside the common ranges reported for granular sludge (Table 9.2 and Figure 9.2). A slight decrease in granule size, biomass concentration and granule density was detected (Table 9.2 and Figure 9.2), this is linked to the wash out of biomass from the reactor during the PNP starvation period, as supported by the time curse solids concentration measured in the effluent (Figure 9.2). In spite of this biomass decrease, nitritation was unaffected and PNP removal recovery after resuming the regular operation of the reactor was fast, as already discussed.

After the total starvation period and recovery of simultaneous nitritation and PNP removal in a continuous aerobic granular reactor, the granular sludge characteristics were not significantly affected by the starvation episode. At the end of the experimental period (day 130), a slight increase in granule size, biomass density and settling velocity was observed (Table 9.2). The increase is linked to the increase of VSS_R during the recovery of biological processes by keeping an appropriate substrate loading to the reactor. Moreover, VSS_E and SVI_5 were consistently remained at a low value below 80 mg L^{-1} and 15 mg g^{-1} TSS, respectively showing the stability of granules. In spite of slight changed of granular sludge characteristics, the performance of simultaneous nitritation and PNP removal in a continuous aerobic granular reactor remained unaffected.

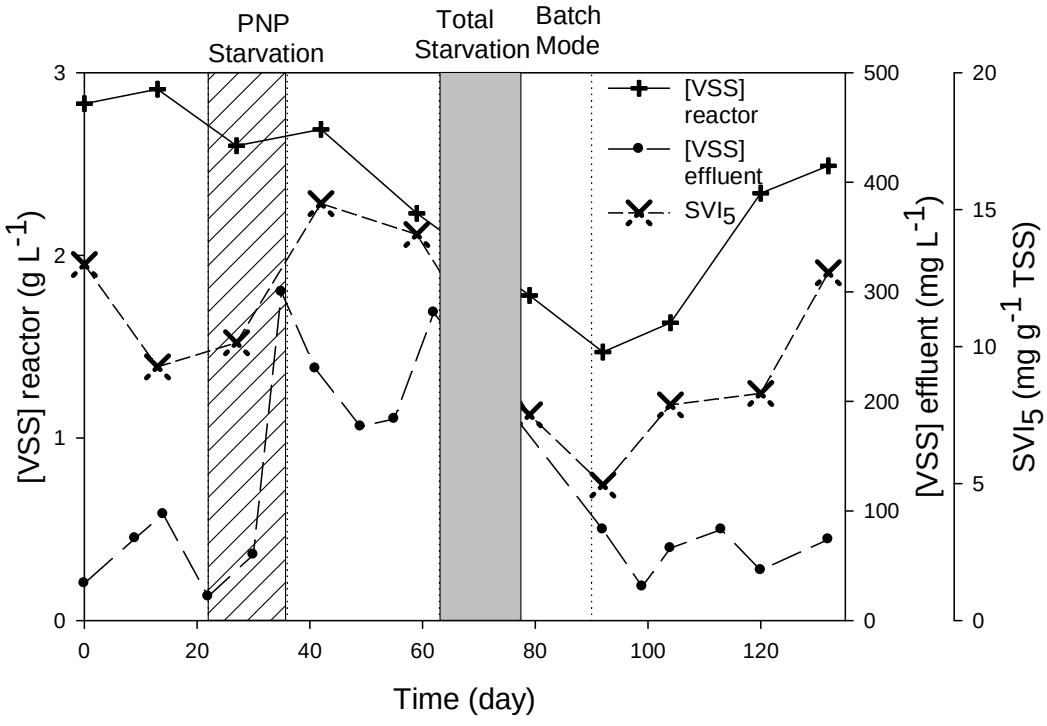

Figure 9.2 The volatile suspended solid (VSS) of biomass in the reactor and in the effluent and SVI_5 during the whole experimental period.

9.4 Conclusions

The following conclusions are drawn:

- The nitritation was unaffected during and after the PNP starvation period showing the reliability and robustness of aerobic granular biomass system.
- By using a continuous aerobic granular reactor, a fast reactivation and recovery of nitritation and PNP removal could be attained after a long-term total starvation ended.
- In spite of slight changed in granular characteristics after the starvation ended, the performance of nitritation and PNP degradation was unaffected and remained identical as before starvation periods.
- Full recovery of nitritation and PNP removal system attained in 21 days by applying a batch mode operation strategy at the beginning and then switching to a continuous operation.

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PART IV

CONCLUSIONS

Chapter 10

Overall conclusions

Summary

In this chapter, the general conclusions and the main achievements of this research are presented. In addition, rooms for future research derived from this thesis are also highlighted.

Initially, the modeling of nitritation in aerobic granular reactor operating in continuous mode was performed. The model was incorporated a [DO]/[TAN] ratio control strategy for full nitritation enhancement. Subsequently, the simultaneous nitritation and phenolic compounds removal using a continuous aerobic granular sludge reactor were demonstrated and performed. The performance and the stability of the reactor were assessed under several operational shocks. Scenarios like shock load events, sequentially alternating pollutant (SAP) and starvation periods were imposed and evaluated.

Modeling of nitritation

The [DO]/[TAN] ratio control strategy implemented in the actual operation of a continuous aerobic granular reactor for obtaining stable nitritation was developed for being included into a mathematical model. Partial and full nitritation conditions were predicted well by the developed model. Aerobic granular sludge reactor performing full nitritation was stably maintained and enhanced when the [DO]/[TAN] control strategy was applied. The model predicted the aerobic granules size larger than 1 mm and higher ammonium concentrations in the influent enhance the achievement of stable full nitritation. Moreover, poor influence of biomass density was predicted with the model.

The model could be utilised for the extension study of obtaining and maintaining long-term operation of full nitritation in aerobic granular reactor operating in continuous mode focusing on:

i. effect of biomass concentration, biofilm surface area and nitrous and nitric oxide emissions
ii. pH variation in the granular reactor
iii. Effect of long starvation periods and subsequent reactivation of nitritation process.

The model also could be extended by coupling with Anammox or heterotrophic denitrification, since suitable effluent for both processes could be obtained without problems by applying [DO]/[TAN] control system.

Simultaneous nitritation and phenolic compounds removal

The feasibility of simultaneous nitritation and phenolic compounds removal was demonstrated in an aerobic granular sludge reactor operating in continuous mode. Fisrt the reactor was performing simultaneous partial nitritation and *o*-cresol removal and second it performed simultaneous nitritation and *p*-nitrophenol (PNP) removal. During most of the experimental period, nitritation was stably maintained and the phenolic compound was removed simultaneously. The unique microbial structure of aerobic granule allows the co-existence of heterotrophic bacteria (that are oxidising phenolic compounds) and autotrophic bacteria (that are oxidising ammonium) within the granule. Thus, the simultaneous removal of ammonium and phenolic compounds using aerobic granular reactors is possible to accomplish. Moreover, the granular reactor performing simultaneous partial nitritation and *o*-cresol removal was exceptionally stable under *o*-cresol shock load events. This stability signifies the robustness and resilient of the aerobic granular sludge under operational uncertainty.

The study of simultaneous removal of complex synthetic wastewater containing ammonium and mixture of phenolic compounds using aerobic granular reactor should be performed. Additionally, the simultaneous removal using real wastewater would also be important to address in the future research.

The continuous aerobic granular reactor performing simultaneous nitritation and phenolic compounds removal at pilot scale should be carried out prior to the implementation at full industrial scale.

The study of substrates and metabolism profile in stratified microbial communities i.e. aerobic granules using microsensors would be essential to understand the biological processes occur inside the granule.

Coupling the simultaneous nitritation and phenolic compounds removal with Anammox or heterotrophic denitrification would be an interesting topic to completely eliminate nitrogen.

Reactor performance under process instability

Several process instability scenarios were performed on the reactors during the simultaneous removal of ammonium and phenolic compounds. During the SAP scenarios, the performance of granular reactor treating simultaneously ammonium and *o*-cresol was stably maintained and unaffected by the presence of PNP or phenol. However, the partial nitritation and *o*-cresol biodegradation were inhibited by the presence of 2-chlorophenol (2CP). The aerobic granular sludge is capable to degrade several phenolic compounds except chlorophenols that are highly inhibitory. Appropriate monitoring and strategy should be applied before allowing highly toxic compounds entering the aerobic granular systems.
The aerobic granular reactor treating simultaneously ammonium and PNP was recovered quickly after long–term starvation periods. The biological process reactivation takes about 11 days to recover its previous performance. Long-term starvation periods do not cause any problem to the granular reactor since the performance of the reactor could be recovered immediately.

Study using a respirometer should be conducted prior to the treatment of toxic compounds in order to gauge the inhibitory impact of those compounds on the simultaneous biological processes. Through respirometer, kinetic parameters could also be determined.

Modeling study describing the simultaneous nitritation and phenolic compounds removal using aerobic granular reactor could be developed. Through the model, several process instability i.e. shock loads, SAP, starvation periods, temperature; could be simulated and investigated.

APPENDICES

A1. Biological Process

Nitrification was defined as a two-step process with a first oxidation of ammonium to nitrite by ammonia-oxidizing bacteria (AOB) and a subsequent oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB).

Stoichiometric and kinetic parameter values together with their corresponding rate expressions are presented in Table A1, Table A2 and Table A3. All the kinetic parameters were taken from literature. A temperature correction was applied through Eq. (A1) to the kinetic parameters corresponding to heterotrophic biomass (i.e., $\mu_{max,H}$, and b_H) as proposed by Henze et al. (2000).

$$k(T) = k(20°C)e^{0.07(T-20°C)} \quad \text{(A1)}$$

where k is $\mu_{max,H}$ or b_H and T is the temperature (^oC).

The values corresponding to the maximum specific growth rates of autotrophic bacteria ($\mu_{max,AOB}$ and $\mu_{max,NOB}$) were calculated with the equations proposed by Jubany et al. (2008):

$$\mu_{max,AOB}(pH,T) = \frac{1.28 \cdot 10^{12} e^{-8183/(273+T)}}{1 + (2.05 \cdot 10^{-9}/10^{-pH}) + (10^{-pH}/1.66 \cdot 10^{-7})} \quad \text{(A2)}$$

$$\mu_{max,NOB}(pH,T) = \frac{6.69 \cdot 10^{7} e^{-5295/(273+T)}}{1 + (2.05 \cdot 10^{-9}/10^{-pH}) + (10^{-pH}/1.66 \cdot 10^{-7})} \quad \text{(A3)}$$

On the other hand, the decay rate expression for both AOB and NOB were calculated with the equations proposed by Munz et al. (2011) and modified with a temperature correction:

$$b_{AER,AOB}(T) = 3.21 \cdot 10^{11} e^{-8183/(273+T)} \quad \text{(A4)}$$

$$b_{ANAER,AOB}(T) = 1.34 \cdot 10^{10} e^{-8183/(273+T)} \quad \text{(A5)}$$

$$b_{AER,NOB}(T) = 9.71 \cdot 10^{6} e^{-5295/(273+T)} \quad \text{(A6)}$$

$$b_{ANAER,NOB}(T) = 4.66 \cdot 10^{5} e^{-5295/(273+T)} \quad \text{(A7)}$$

The terms TAN and TNN were used instead of ammonium and nitrite because they are the true compounds analyzed in the chemical analyses. Eqs. (A8) and (A9), derived from acid-base equilibriums, were used for the calculation of the free ammonia (FA or NH_3) and the free nitrous acid (FNA or HNO_2) concentrations in equilibrium with TAN and TNN, respectively.

$$FA = \frac{TAN \cdot 10^{pH}}{\frac{K_b}{K_w} + 10^{pH}} \cdot \frac{14}{17} \quad \text{(A8)}$$

$$FNA = \frac{TNN}{K_a \cdot 10^{pH} + 1} \cdot \frac{47}{14} \quad \text{(A9)}$$

The ratio between the ionization constant of the ammonia equilibrium (K_b) and the ionization constant of water (K_w) is related to the temperature as shown in Eq. (A10) and the temperature effect on the ionization constant of the nitrous acid equilibrium (K_a) is shown in Eq. (A11) (Anthonisen et al., 1976)

$$\frac{K_b}{K_w} = \exp\left(\frac{6344}{273 + T}\right) \quad \text{(A10)}$$

$$K_a = \exp\left(\frac{-2300}{273 + T}\right) \quad \text{(A11)}$$

The kinetics for each of the processes considered i.e. growth and decay of each kind of bacteria are shown in Table A2. In addition, the oxygen limitations, substrate and non-competitive inhibitions for AOB and NOB growth processes were also considered. AOB inhibition by TAN and NOB inhibition by TNN were described with a Haldane model while AOB inhibition by TNN and NOB inhibition by TAN were described with a non-competitive model.

Table A1. Stoichiometric Matrix

j	Process	S_{O_2}	S_{TAN}	S_{TNN}	S_{NO_3}	S_S	X_{AOB}	X_{NOB}	X_H	X_I
1	Growth of X_{AOB}	$-(3.43-Y_{AOB})/Y_{AOB}$	$-1/Y_{AOB}$	$1/Y_{AOB}$			1			
2	Decay of X_{AOB}						-1			1
3	Growth of X_{NOB}	$-(1.14-Y_{NOB})/Y_{AOB}$		$-1/Y_{NOB}$	$1/Y_{NOB}$			1		
4	Decay of X_{NOB}							-1		1
5	Aerobic growth of X_H	$-(1-Y_H)/Y_H$				$-1/Y_H$			1	
6	Decay of X_H								-1	1
	Units	g O_2 m^{-3}	g N m^{-3}	g N m^{-3}	g N m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}

Table A2. Kinetic rate expressions

j	Process	Process rate (d^{-1})	Reference
1	Growth of X_{AOB}	$\mu_{\max,AOB} \cdot \frac{S_{O_2}}{K_{O_2,AOB} + S_{O_2}} \cdot \frac{S_{TAN}}{K_{S,TAN,AOB} + S_{TAN} + \frac{S_{TAN}^{\ 2}}{K_{I,TAN,AOB}}} \cdot \frac{K_{I,TNN,AOB}}{K_{I,TNN,AOB} + S_{TNN}} \cdot X_{AOB}$	Jubany et al. (2008)
2	Decay of X_{AOB}	$b_{AER,AOB} \cdot \frac{S_{O_2}}{K_{O_2,AOB} + S_{O_2}} \cdot X_{AOB} + b_{ANAER,AOB} \cdot X_{AOB}$	Munz et al. (2011)
3	Growth of X_{NOB}	$\mu_{\max,NOB} \cdot \frac{S_{O_2}}{K_{O_2,NOB} + S_{O_2}} \cdot \frac{S_{TNN}}{K_{S,TNN,NOB} + S_{TNN} + \frac{S_{TNN}^{\ 2}}{K_{I,TNN,AOB}}} \cdot \frac{K_{I,TAN,NOB}}{K_{I,TAN,NOB} + S_{TAN}} \cdot X_{NOB}$	Jubany et al. (2008)
4	Decay of X_{NOB}	$b_{AER,NOB} \cdot \frac{S_{O_2}}{K_{O_2,NOB} + S_{O_2}} \cdot X_{NOB} + b_{ANAER,NOB} \cdot X_{NOB}$	Munz et al. (2011)
5	Growth of X_H	$\mu_{\max,H} \cdot \frac{S_{O_2}}{K_{O_2,H} + S_{O_2}} \cdot \frac{S_S}{K_{S,H} + S_S} \cdot X_H$	Henze et al. (2000)
6	Decay of X_H	$b_H \cdot X_H$	Henze et al. (2000)

Table A3. Kinetic parameters (30 ^{o}C and pH 8.2)

Symbol	Definition	Value	Unit	References
	Ammonia oxidizing bacteria (AOB)			
$\mu_{max,AOB}$	Maximum specific growth rate of X_{AOB}	2.3	d^{-1}	Jubany et al. (2008)
$b_{AER,AOB}$	Decay rate	0.6	d^{-1}	Munz et al. (2011)
$b_{ANAER,AOB}$	Anaerobic decay rate	0.025	d^{-1}	Munz et al. (2011)
Y_{AOB}	Growth yield	0.18	g COD g^{-1} N	Jubany et al. (2008)
$K_{O2,AOB}$	Affinity constant for oxygen	0.74	mg O_2 L^{-1}	Guisasola et al. (2005)
$K_{S,TAN}$	Affinity constant for TAN	11	mg TAN L^{-1}	Carrera et al. (2004)
$K_{I,TAN,AOB}$	Inhibition coefficient for TAN	675	mg TAN L^{-1}	Jubany et al. (2009)
$K_{I,TNN,AOB}$	Inhibition coefficient for TNN	13115	mg TNN L^{-1}	Jubany et al. (2009)
	Nitrite-oxidizing bacteria (NOB)			
$\mu_{max,NOB}$	Maximum specific growth rate of X_{NOB}	1.65	d^{-1}	Jubany et al. (2008)
$b_{AER,NOB}$	Decay rate	0.25	d^{-1}	Munz et al. (2011)
$b_{ANAER,NOB}$	Anaerobic decay rate	0.012	d^{-1}	Munz et al. (2011)
Y_{NOB}	Growth yield	0.08	g COD g^{-1} N	Jubany et al. (2008)
$K_{O2,NOB}$	Affinity constant for oxygen	1.75	mg O_2 L^{-1}	Guisasola et al. (2005)
$K_{S,TNN}$	Affinity constant for TNN	22	mg TNN L^{-1}	Ni et al. (2008)
$K_{I,TNN,NOB}$	Inhibition coefficient for TNN	1431	mg TNN L^{-1}	Jubany et al. (2008)
$K_{I,TAN,NOB}$	Inhibition coefficient for TAN	145	mg TAN L^{-1}	Brockmann and Morgenroth (2010)
	Heterotrophic bacteria (H)			
$\mu_{max,H}$	Maximum specific growth rate of X_H	11.8	d^{-1}	Henze et al. (2000)
b_H	Decay rate	7.87	d^{-1}	Henze et al. (2000)
Y_H	Growth yield	0.67	g COD g^{-1} N	Henze et al. (2000)
$K_{O2,H}$	Affinity constant for oxygen	0.2	mg O_2 L^{-1}	Henze et al. (2000)
$K_{S,S}$	Affinity constant for substrate	4	mg COD L^{-1}	Henze et al. (2000)

Table A4. Diffusivity coefficients

Parameter	Symbol	Value	Unit	References
Diffusivity of O_2 in water	D_{O2}	$2.2 \cdot 10^{-4}$	m^2 d^{-1}	Picioreanu et al. (1997)
Diffusivity of NH_4^+ in water	D_{TAN}	$1.9 \cdot 10^{-4}$	m^2 d^{-1}	Picioreanu et al. (1997)
Diffusivity of NO_2^- in water	D_{TNN}	$1.7 \cdot 10^{-4}$	m^2 d^{-1}	Picioreanu et al. (1997)
Diffusivity of NO_3^- in water	D_{NO3}	$1.7 \cdot 10^{-4}$	m^2 d^{-1}	Picioreanu et al. (1997)
Diffusivity of organic substrate in water	D_S	$1.0 \cdot 10^{-4}$	m^2 d^{-1}	Picioreanu et al. (1997)
Diffusivity coefficient inside biofilm	E_{diff}	0.5	dimensionless	Assumed, in the range proposed by Bishop et al. (1995)

A2. AQUASIM implementation

Further details describing the AQUASIM implementation of closed-loop control of ammonium concentration are given in Fig. A1 and Eqs. A12-13.

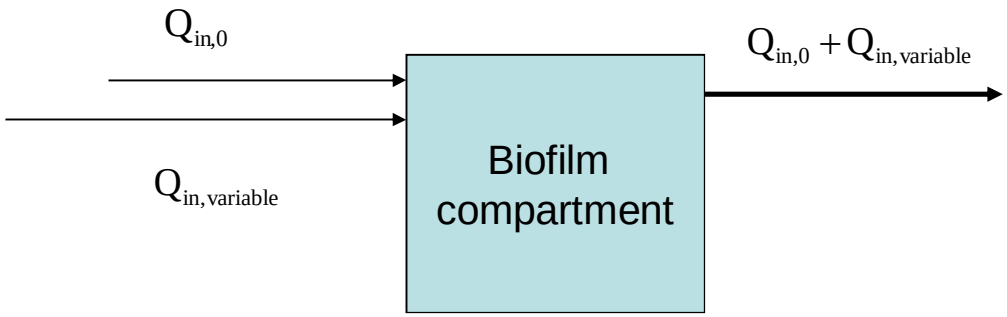

Figure A1. Schematic diagram to illustrate the implementation of the closed-loop control of ammonium in AQUASIM. Flow rates are detailed in Eq. 4.2 and Eq. A12-A13.

$$Q_{in,variable} = Q_{in,0} . \frac{[TAN]_{SP} - [TAN]}{[TAN]_{SP}} . a \qquad \text{(A12)}$$

$$Q_{in} = Q_{in,0} + Q_{in,variable} \qquad \text{(A13)}$$

A3. Highlighting the effects of closed-loop control

In addition to Figs. 4.2-4.4 already presented to highlight the effects of closed-loop control, simulations were run applying the same TAN concentration disturbances, but keeping a constant value for the DO/TAN concentration ratio in the reactor by proper manipulation of the DO concentration. This simulation was perform for both scenarios (monthly and daily disturbances), and in both cases full nitritation was always obtained, showing the efficiency of the ratio control strategy (see Figs. A2 and A3).

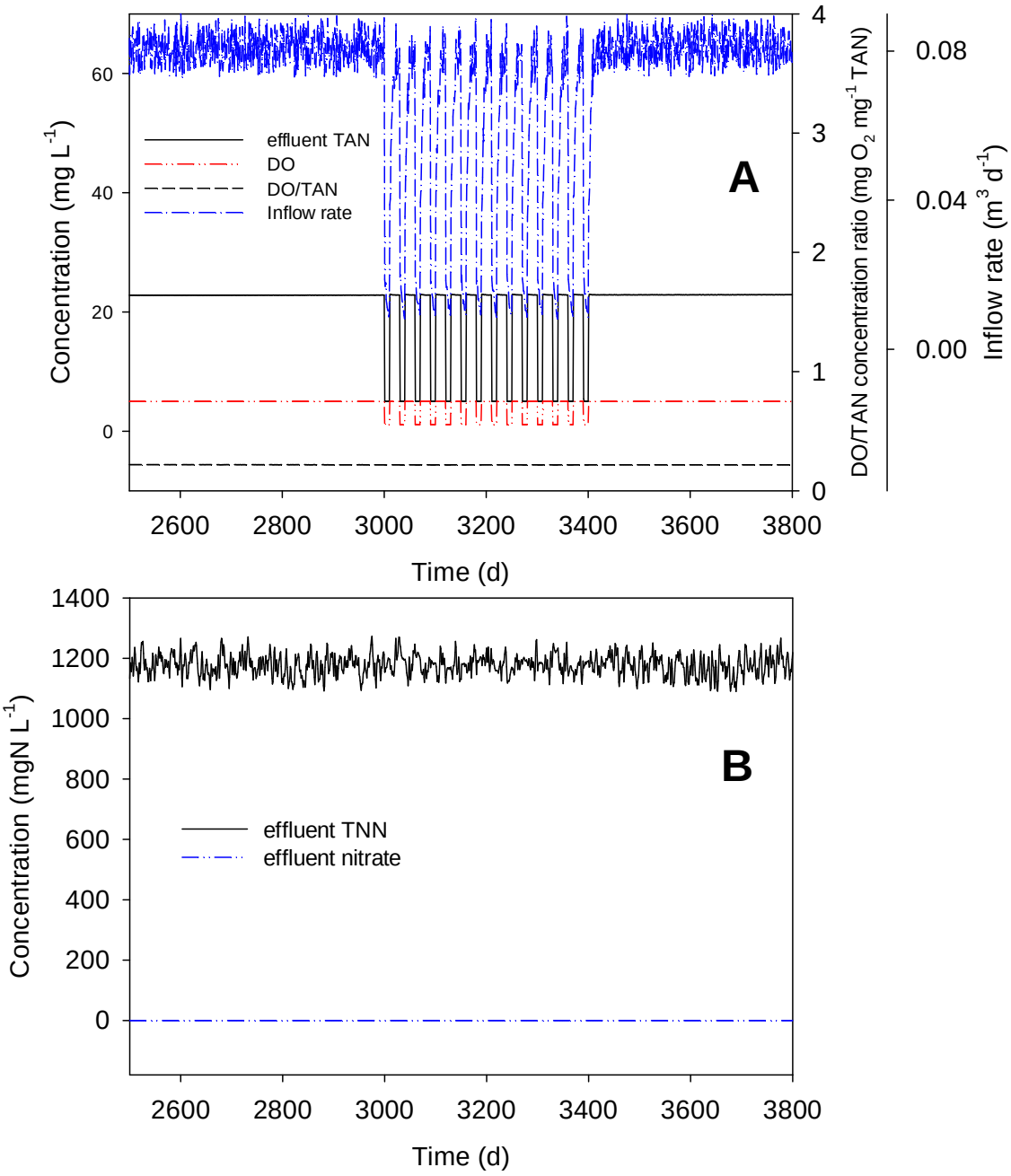

Figure A2. Ratio control on a monthly basis (scenario 1). TAN concentration decreased from 23 to 5 mg N L^{-1} periodically (one third of the month at low TAN concentration) during 400 days. Note how DO concentration was manipulated conveniently to keep a constant value of the DO/TAN concentration ratio at 0.22 mgO_2 mg^{-1} TAN. All data plotted correspond to modeling results.

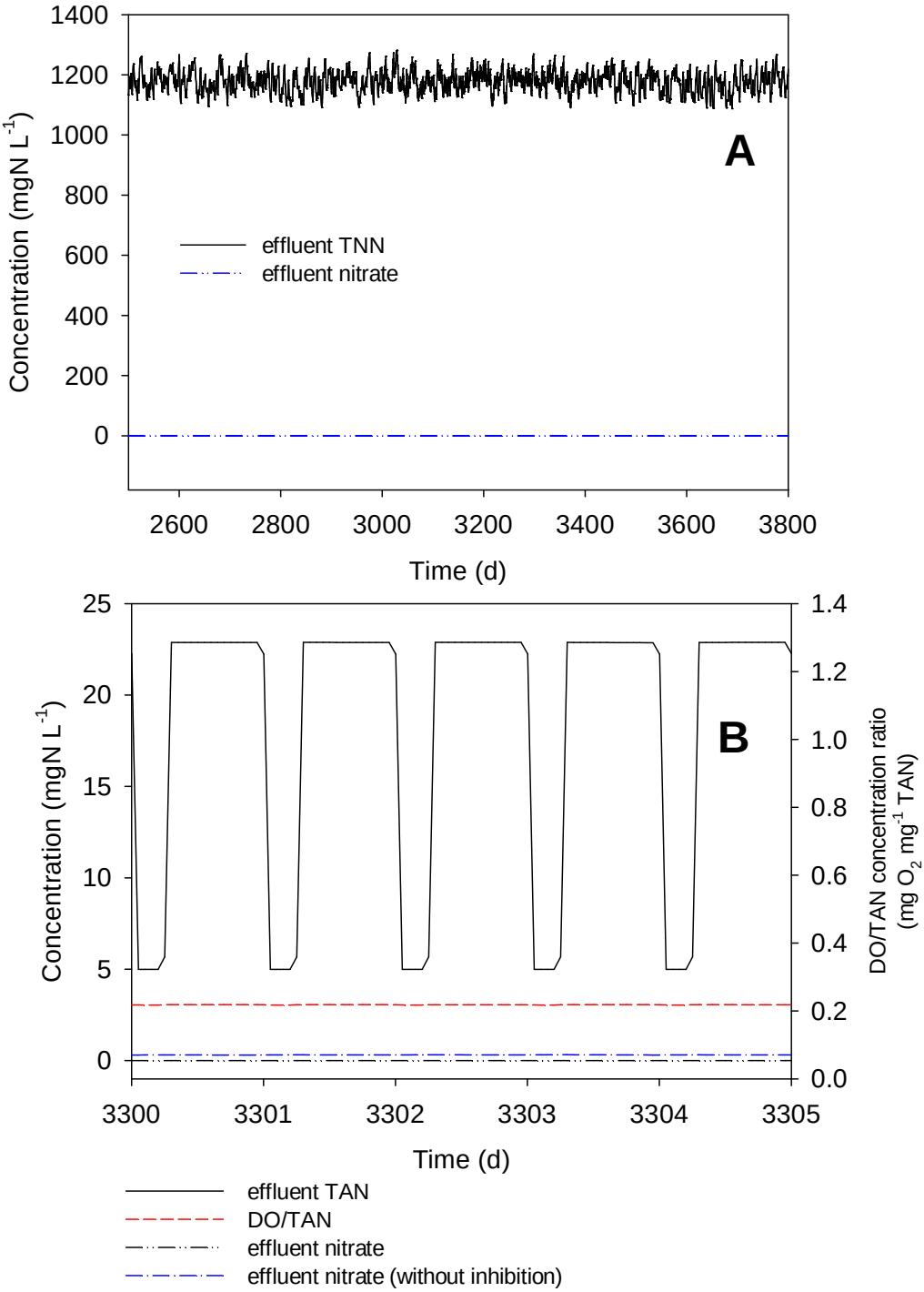

Figure A3. Ratio control on a daily basis (scenario 2). TAN concentration decreased from 23 to 5 mg N L^{-1} periodically (one third of the day at low TAN concentration) during 400 days (since day 3000 until day 3400). B: zommed in graph of Fig. A2 (A) to show the effect of the DO/TAN ratio on nitritation. The simulation was also computed considering no inhibition of AOB and NOB by free ammonia ($K_{I,TAN,AOB}$ = $K_{I,TAN,NOB}$ = 10^4 mg N L^{-1}). Note how DO concentration was manipulated conveniently to keep a constant value of the DO/TAN concentration ratio at 0.22 mgO_2 mg^{-1} TAN. All data plotted correspond to modeling results.

A4. Effect of the DO/TAN concentration ratio

Further details about the simulation results used to build Fig. 4.5 are given below in Table A5.

Table A5. Specific setpoints imposed for the simulations presented in Fig. 4.5. Outputs of the model are shown for an easier understanding of how the results presented in Fig. 4.5 were found. Besides the TAN_{SP} and R_{SP}, the values for TAN concentration and R found in steady state as determined with the mathematical model are also presented. DO concentration in steady state was always very close to setpoint, so for practical purposes both values can be considered equal.

	Model inputs			Model outputs				
	$[TAN]_{SP}$ (mg N L^{-1})	R_{SP} (mg O_2 mg^{-1} TAN)	$[DO]_{SP}$ (mg N L^{-1})	[TAN] (mg N L^{-1})	R (mg O_2 mg^{-1} TAN)	NLR_V (g N L^{-1} d^{-1})	TAN converted to TNN (%)	TAN converted to NO_3^- (%)
Figure 4.5A	80	0.063	5	79.44	0.0629	1.2407	93.50	0.00
	50	0.100	5	49.7	0.1007	1.1636	96.00	0.00
	35	0.143	5	34.8	0.1437	1.0532	97.25	0.00
	25	0.200	5	24.9	0.2010	0.9075	98.42	0.00
	20	0.250	5	19.9	0.2511	0.7886	98.50	0.00
	19	0.263	5	18.9	0.2643	0.7982	12.23	86.33
	18	0.278	5	17.9	0.2789	0.7661	7.63	91.00
	15	0.333	5	14.9	0.3345	0.6514	3.55	95.33
	10	0.500	5	9.98	0.5009	0.3611	1.23	98.08
	8	0.625	5	7.99	0.6255	0.1886	0.77	98.75
Figure 4.5B	20	0.650	13	19.90	0.6533	1.0939	5.63	92.83
	20	0.600	12	19.90	0.6030	1.0725	5.90	92.58
	20	0.550	11	19.90	0.5528	1.0479	6.14	92.33
	20	0.500	10	19.90	0.5025	1.0243	6.93	91.50
	20	0.450	9.0	19.90	0.4523	0.9954	7.77	90.67
	20	0.400	8.0	19.90	0.4020	0.9621	8.51	89.92
	20	0.350	7.0	19.90	0.3518	0.9225	9.03	89.42
	20	0.340	6.8	19.90	0.3417	0.8807	98.42	0.00
	20	0.330	6.6	19.90	0.3317	0.8711	98.42	0.00
	20	0.315	6.3	19.91	0.3164	0.8571	98.42	0.00
	20	0.300	6.0	19.90	0.3015	0.8421	98.42	0.00
	20	0.250	5.0	19.90	0.2513	0.7886	98.42	0.00
	20	0.200	4.0	19.90	0.2010	0.7232	98.42	0.00
	20	0.150	3.0	19.90	0.1508	0.6407	98.42	0.00
	20	0.100	2.0	19.90	0.1005	0.5304	98.42	0.00
	20	0.050	1.0	19.96	0.0501	0.3600	98.42	0.00

A5. Effect of the granule size

For Section 4.4.4 *Effect of granule size on* $R_{max,fN}$, with main results presented in Fig. 4.6, the granule size was considered to vary in the range [0.05-3] mm. In the estimation of biofilm surface the experimental values for steady state C were used, following the

procedure defined in Eqs. 4.3-4.5. The total biofilm surface area was kept constant varying the number of granules for each granule size, as specified in Table A6.
For each value in Fig. 4.6, an iterative procedure to find $R_{max,fN}$ was followed, see a description in Fig. A4.

Table A6. Numerical examples showing how the number of granules was selected to keep a total biofilm area in the reactor in the simulations presented in Fig. 4.6.

Granule size (mm)	Number of granules	Total biofilm area (m^2)
0.05	$1.5 \cdot 10^9$	11.8
0.5	$1.5 \cdot 10^7$	11.8
1.5	$1.7 \cdot 10^6$	11.8
2.5	$6.0 \cdot 10^5$	11.8
3.0	$4.2 \cdot 10^5$	11.8

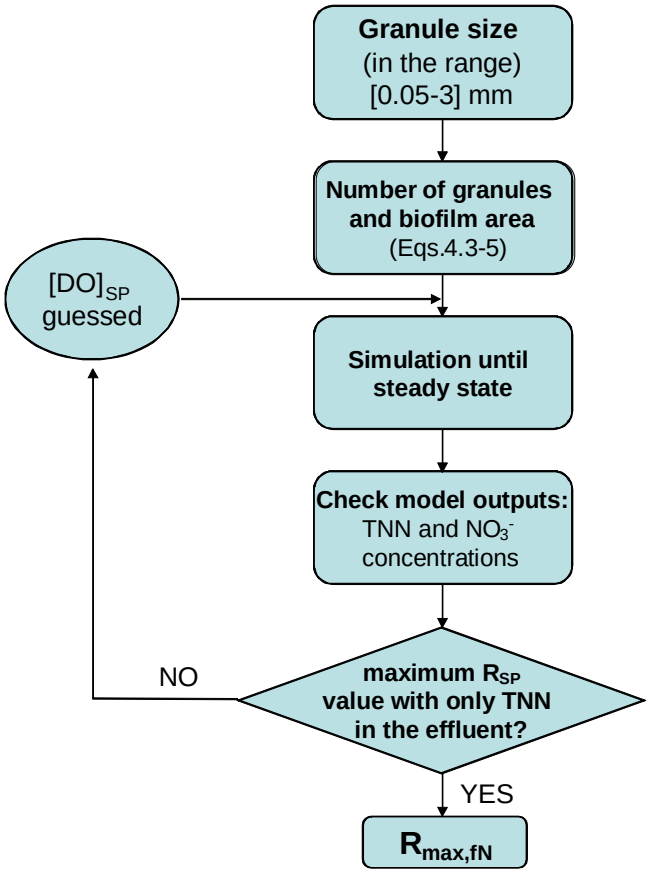

Figure A4. Flow diagram to illustrate the iterative procedure used to determine $R_{max,fN}$ for each granule size selected.

A.6 References

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B.1 Determination of the PNP-degraders half saturation coefficient for oxygen, $K_{O_{PNP}}$.

The methodology to determine the $K_{O_{PNP}}$ was following Guisasola et al. (2005). As can be deduced, the $K_{O_{PNP}}$ represents the dissolved oxygen (DO) concentration at which the *p*-nitrophenol (PNP) biodegradation rate is half of its maximum rate. The biodegradation kinetics dependence on the DO concentration is generally described using Monod expression (Eq. B1).

$$r = r_{max} \frac{[DO]}{K_{OPNP}+[DO]} \qquad \text{Eq. B1}$$

where;

r = PNP degradation rate (mg PNP mg $VSS^{-1}d^{-1}$)
r_{max} = maximum PNP degradation rate (mg PNP mg $VSS^{-1}d^{-1}$)
[DO] = dissolved oxygen concentration (mg O_2 L^{-1})
$K_{O_{PNP}}$ = PNP-degraders affinity constant for DO (mg O_2 L^{-1})

B1.1 Methods

An open and aerated LFS respirometer was employed in this study. The respiration vessel's volume was 0.8 L and it was magnetically stirred. pH, DO 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 27 ± 0.3 °C. The pH was maintained at 8.0 ± 0.1 with controlled addition of HCl (1M) and NaOH (1M).

B1.2 Procedure

The activated sludge enriched with PNP-degrading bacteria was employed in this study (Martín-Hernández et al., 2009). The procedure involved the monitoring of DO drop in the respirometric vessel when the aeration was turned off and the biomass was consuming without substrate (PNP) limitations. A pulse of substrate (10 mg PNP L^{-1}) was added to the respirometer and once the maximum rate was reached, the aeration was stopped. At this moment, the DO 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) assuming that no substrate limitations existed. It was essential to avoid any substrate limitation (except for oxygen) for a reliable $K_{O_{PNP}}$ estimation. The PNP degradation rate decreased as the DO concentration also decreased in the respirometer due to oxygen limitations. The lower the DO level was, the more important the oxygen limitation effect became (Eq. B1). Figure B1 illustrates the typical DO concentration profile observed and the main procedure used and in the determination of $K_{O_{PNP}}$. The following expression was used to describe the system:

$$\frac{dS_o}{dt} = k_L a \cdot [S_o^* - S_o(t)] - (OUR_{end} + OUR_{max}) \cdot \frac{S_o}{K_{OPNP}+S_o} \qquad \text{Eq. B2}$$

where;

k_La = global oxygen mass transfer constant (d^{-1})
S_o^* = dissolved oxygen saturation level (mg O_2 L^{-1})
S_o = dissolved oxygen concentration (mg O_2 L^{-1})
OUR_{end} = endogenous OUR value (mg O_2 $L^{-1}d^{-1}$)

$$OUR_{max} = \frac{1.44-Y}{Y} \cdot \mu_{max} \cdot X \quad \text{Eq. B3}$$

where

Y = the biomass yield (mg COD mg^{-1} PNP)
X = the biomass concentration (mg COD L^{-1}), considering the biomass as $C_5H_7NO_2$ for volatile suspended solid (VSS) conversion
μ_{max} = the maximum specific growth rate (d^{-1})

All parameters were modeled, simulated and estimated using MATLAB 7.5 (The Mathworks, Natick, MA). The differential equations were solved using an explicit Runge-Kutta formula. Parameter estimation was carried out by using the Nelder-Mead Simplex search method. Further detail can be found in Guisasola et al. (2005).

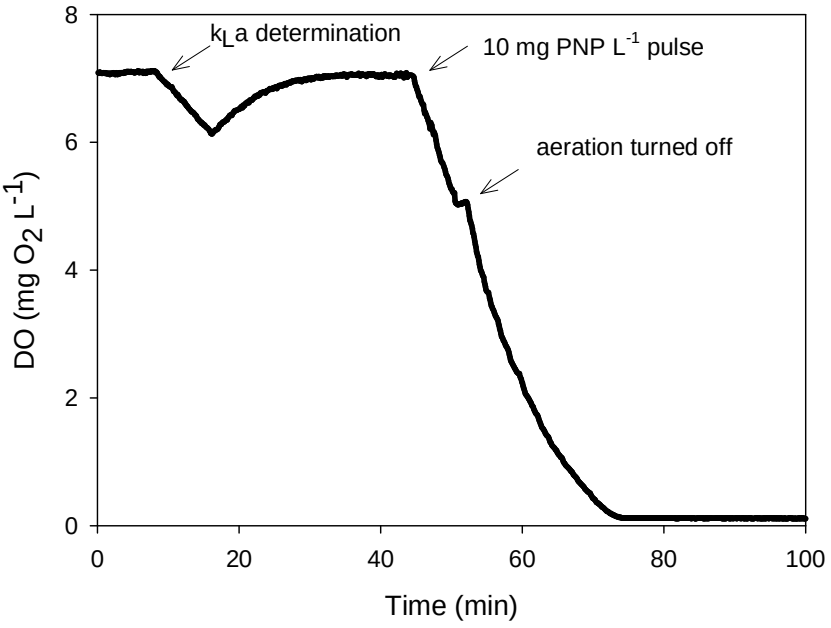

Figure B1. The dissolve oxygen (DO) profile obtained in determining the PNP-degraders half saturation coefficient for oxygen, $K_{O_{PNP}}$. Experimental conditions were temperature, 27 ± 0.3 °C; pH, 8.0 ± 0.1.

B1.3 Results

The estimated $K_{O_{PNP}}$ value is 1.65 ± 0.01 mg O_2 L^{-1}.

B.2 PNP inhibition coefficient over AOB, $K_{I_{PNP,AOB}}$

The experimental protocol for the $K_{I_{PNP,AOB}}$ determination was according to the method described by Guisasola et al. (2003) and Suárez-Ojeda et al. (2007). In this method, the inhibition percentage was calculated by comparing the maximum OUR measured in the control pulses (ammonium) before and after the addition of inhibitory substrates (PNP). The following equation described the calculation (Suárez-Ojeda et al., 2010):

$$\%\, Inhibition = \left[\frac{OUR_{max}^{1st\ control\ pulse} - OUR_{max}^{2nd\ control\ pulse}}{OUR_{max}^{1st\ control\ pulse}}\right] \cdot 100 \qquad \text{Eq. B4}$$

The OUR profile was obtained by solving the dissolved oxygen balance in the liquid phase through a respirometric experiment. Assuming constant liquid volume and distinguishing between the OUR due to the endogenous process from the OUR due the exogenous process (OUR_{EX}), the following equation can be obtained:

$$OUR_{EX}(t) = k_L a[S_o^* - DO(t)] - \frac{dDO(t)}{dt} \qquad \text{Eq. B5}$$

where, k_La of the reactor was estimated using the experimental reaeration profile obtained by turning off the aeration and subsequently turning it on.

In addition, a non-competitive inhibition model was used to determined $K_{I_{PNP,AOB}}$:

$$\%\, Inhibition = \left(1 - \frac{K_{iPNP,AOB}}{K_{iPNP,AOB} + [I]}\right) \cdot 100 = \qquad \text{Eq. B6}$$

where [I] is the inhibitor concentration (mgL^{-1}) and $K_{I_{PNP,AOB}}$ is the inhibition coefficient (mgL^{-1}). In this case the $K_{I_{PNP,AOB}}$ value equals to the inhibitor concentration resulting in a 50% inhibition (IC_{50}) of AOB activity.

B.2.1 Methods

An open and aerated LFS respirometer was employed in this study. All the set-up of the experiment was similar as previously described in Section B.1.1. The temperature was maintained at 27 ± 0.3 °C and the pH was maintained at 7.5 ± 0.1 with controlled addition of HCl (1M) and NaOH (1M).

B.2.2 Procedure

An activated sludge enriched with AOB (Torà et al., 2012) was used for the inhibitory effect study of PNP over nitrititation. Ammonium (10 mg N L^{-1}) was used as a control substrate and different concentrations of PNP 0.5, 2, 10, 15, 25 and 30 mgL^{-1} were used as inhibitory substrate. The OUR profile obtained was used for the estimation of inhibition percentage. Each test consisted in three sequential pulses: a first control pulse of 10 mg L^{-1} of ammonium, a pulse of the PNP and finally a second control pulse added after the DO concentration return to its initial saturation DO (the DO before the first control substrate was added).

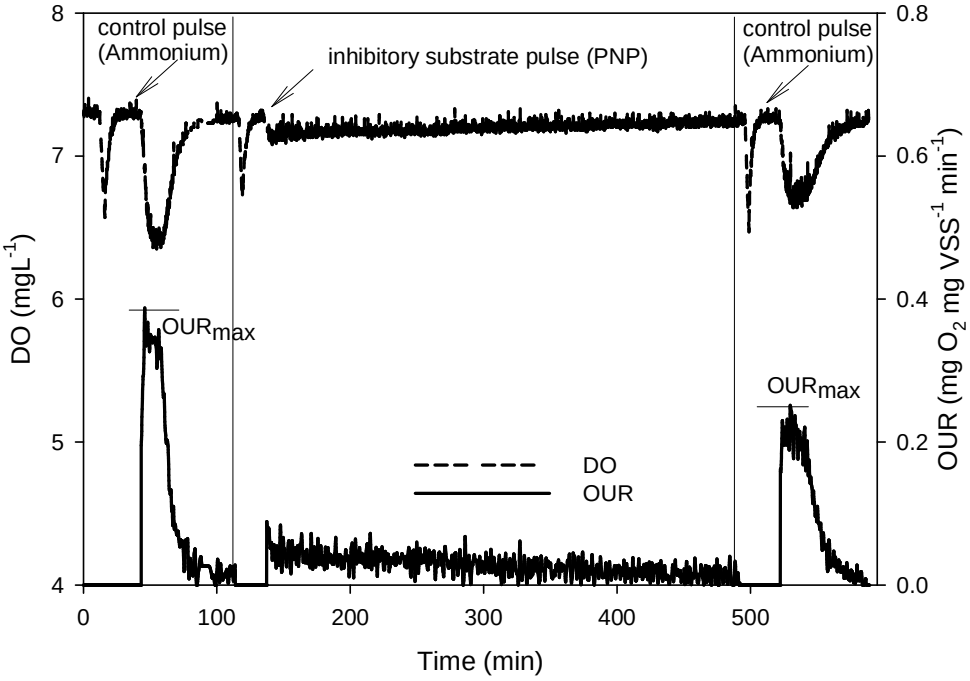

Figure B2 The DO profile obtained from the respirometric test for the determination of PNP inhibitory coefficient over AOB. The calculated oxygen uptake rate (OUR) was also depicted in the figure. Experimental conditions were temperature, 27 ± 0.3 °C; pH, 7.5 ± 0.1; Ammonium, 10 mg N L^{-1}; PNP, 10 mg L^{-1}.

B.2.3 Results

The results of the inhibitory study are presented in the following Figure B3.

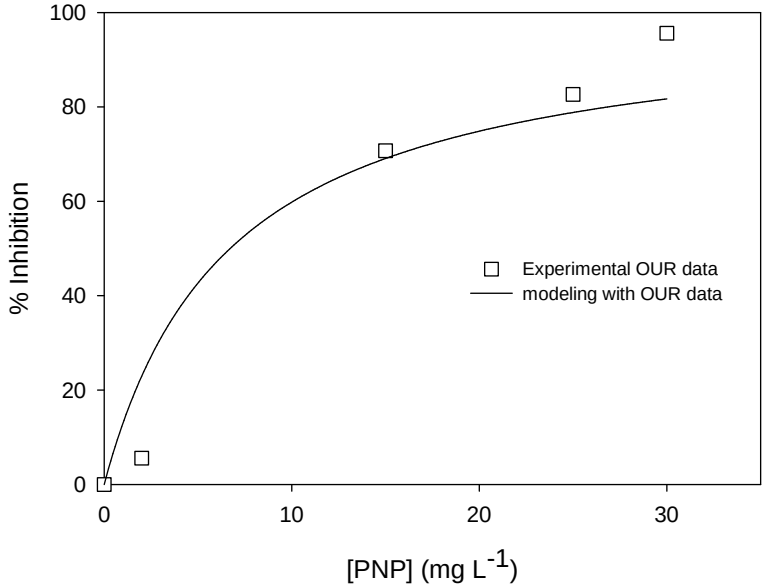

Figure B3 Experimental and simulated inhibition values for PNP over AOB

By using Eq. B6, the inhibition coefficient, $K_{I_{PNP,AOB}}$ value of 7 ± 2 mg L^{-1} for PNP over AOB was obtained.

B.3 References

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