SIMULTANEOUS REMOVAL OF NUTRIENTS AND ENDOCRINE DISRUPTING CHEMICALS BY AEROBIC GRANULAR SLUDGE AND ADVANCED OXIDATION PROCESSES

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REMOÇÃO SIMULTÂNEA DE NUTRIENTES E DESREGULADORES ENDÓCRINOS UTILIZANDO LODO GRANULAR AERÓBIO E PROCESSOS DE OXIDAÇÃO AVANÇADOS

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Agosto/2020

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Programa: Engenharia Química

Esse trabalho de pesquisa teve como objetivo avaliar a remoção de matéria orgânica, nutrientes e desreguladores endócrinos (DE), particularmente 17β-estradiol (E2) e 17α-etinilestradiol (EE2), de matrizes aquosas sintéticas, utilizando os processos de lodo granular aeróbio (LGA) e fotocatálise heterogênea em micro reator de membrana com permeação contínua de H_2O_2. O trabalho experimental foi separado em três etapas. Na primeira, avaliou-se o efeito do tempo de retenção de sólidos (TRS) na estabilidade e desempenho de um reator de LGA ao longo de 392 dias. Maiores eficiências de remoção de DQO, amônia e fósforo total (acima de 93%) foram observadas para um TRS de 15 dias. Na segunda etapa, acompanhou-se o desempenho do reator durante 151 dias tanto em termos de remoção de DQO, NH_4^+, e PO_4^{3-}, como dos DE, sendo também avaliada a atividade estrogênica no efluente. Os resultados mostraram que o sistema foi capaz de atingir elevadas remoções de DQO (93%), NH_4^+ (87%) e PO_4^{3-} (87%), com percentual de biodegradação dos DE acima de 90%, enquanto níveis moderados de atividade estrogênica foram detectados no efluente do reator (0.122 μg L^{-1} EQ-E2). Finalmente, na terceira etapa, investigou-se a remoção dos DE por fotocatálise heterogênea no micro reator de membrana. O sistema UVC/H_2O_2/TiO_2 foi o que apresentou a maior capacidade de oxidação dos DE, com remoções em torno de 50% para a matriz sintética avaliada.

Abstract of Thesis presented to COPPE/UFRJ as a partial fulfillment of the requirements for the degree of Doctor of Science (D.Sc.)

SIMULTANEOUS REMOVAL OF NUTRIENTS AND ENDOCRINE DISRUPTING CHEMICALS BY AEROBIC GRANULAR SLUDGE AND ADVANCED OXIDATION PROCESSES

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This research work aimed to evaluate the removal of organic matter, nutrients and endocrine disrupting chemicals (EDCs), particularly 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), from synthetic aqueous matrices, using aerobic granular sludge (AGS) and heterogeneous photocatalysis in a membrane micro-reactor with continuous H_2O_2 permeation. The experimental work was separated into three stages. In the first, the effect of solids retention time (SRT) on the stability and performance of an AGS reactor over 392 days was evaluated. Higher COD, ammonia and total phosphorus removal efficiencies (above 93%) were observed at an SRT of 15 days. In the second stage, the performance of the reactor was monitored for 151 days, both in terms of COD, NH_4^+, and PO_4^{3-} removal, and DE abatement, while the estrogenic activity was assessed in the effluent. The results showed that the system was able to achieve high removals of COD (93%), NH_4^+ (87%) and PO_4^{3-} (87%), with a EDCs biodegradation percentage above 90%, while moderate levels of estrogenic activity were detected in the reactor effluent samples (0.122 μg L^{-1} EQ-E2). Finally, in the third stage, the removal of EDCs was investigated by heterogeneous photocatalysis in the membrane micro reactor. The UVC/H_2O_2/TiO_2 system was the one that showed the highest oxidation capacity of EDCs, with a removal of around 50% for the synthetic matrix evaluated.

SUMMARY

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LIST OF SYMBOLS AND ABBREVIATIONS

AGS	Aerobic Granular Sludge
AMO	Ammonia Monooxygenase
AOB	Ammonia-oxidizing Bacteria
AOP	Advanced Oxidation Processes
ARZ	Annular Reaction Zone
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
BPR	Back Pressure Regulator
CECs	Contaminants of Emerging Concern
CNPq	*Conselho Nacional de Desenvolvimento Científico e Tecnológico* (National Council for Scientific and Technological Development)
COD	Chemical Oxygen Demand
CONAMA	*Conselho Nacional do Meio Ambiente* (National Environment Council)
CPRG	Chlorophenol Red-β-galactopyraneside
DAD	Diode Array Detector
DGAO	Denitrifying Glycogen-Accumulating Organism
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DPAO	Denitrifying Phosphate-Accumulating Organism
E1	Estrone
E2	17β-estradiol
E3	Estriol
EBPR	Enhanced Biological Phosphorus Removal
EC_{50}	Concentration that elucidates 50% of the estrogenic activity
EDC	Endocrine Disrupting Chemical
EDTA	Ethylenediaminetetraacetic Acid
EE2	17α-ethinylestradiol
EPS	Extracellular Polymeric Substances
EQ-E2	Estradiol Equivalent
FISH	Fluorescence in Situ Hybridization

F/M	Food-to-Microorganism Ratio
FSS	Fixed Suspended Solids
GAO	Glycogen Accumulating Organism
H_2O_2	Hydrogen Peroxide
HO•	Hydroxyl Radicals
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
IC	Inorganic Carbon
K_{OC}	Adsorption Coefficient
K_{OW}	Octanol-Water Partition Coefficient
LabPol	*Laboratório de Controle de Poluição das Águas* (Water Pollution Control Laboratory)
MBBR	Moving Bed Biofilm Reactor
NH_3-N	Ammoniacal Nitrogen
NH_4^+-N	Ammonium Nitrogen
NADH	Nicotinamide Adenine Dinucleotide
NEM	Nano-Engineered Membrane
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_X	Oxidized Nitrogen Compounds
NOB	Nitrite-Oxidizing Bacteria
NOM	Natural Organic Matter
NTU	Nephelometric Turbidity Units
PAO	Polyphosphate Accumulating Organism
PBS	Phosphate-Buffered saline
PCPs	Personal Care Products
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PLC	Programmable Logic Controller
PO_4^{3-}-P	Orthophosphate as Phosphorus
POPs	Persistent Organic Pollutants
PN	Total Proteins
PS	Total Polysaccharides
PW	Pure Water

SBR	Sequencing Batch Reactor
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SND	Simultaneous Nitrification and Denitrification
SPE	Solid Phase Extraction
SRT	Solid Retention Time
SVI	Sludge Volume Index
TiO_2	Titanium Dioxide
TN	Total Nitrogen
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket
UV	Ultraviolet
UWW	Urban Wastewater
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant
Y	Biomass Yield Coefficient
YES	Yeast Estrogen Screen

1. Introduction and Objectives

The water quality is a major issue nowadays, since it involves a large part of the population that uses this vital resource for their survival. The improper discharge of wastewaters from urban, industrial and agricultural activities without a previous treatment is one of the biggest problems that affects the quality of water bodies. In most cases, the wastewaters are composed of complex mixtures whose chemical characterization requires a long time and involves high costs (DEZOTTI, 2008). The main pollutants found in sewers are: organic and/or inorganic matter in solution (biodegradable or not), colloidal matter and suspended solids.

One of the main consequences of untreated wastewaters disposal into receiving bodies is the eutrophication. This process occurs due to the high levels of nutrients, mainly nitrogen and phosphorus, present in waste streams without the proper treatment. Nitrogen and phosphorus are essential nutrients for the growth of microorganisms. However, when these nutrients are in excess, they cause an excessive proliferation of primary producers, such as algae and phytoplankton, which can cause toxicological effects and an imbalance of the aquatic environment.

Result of the eutrophication process, the high concentration of primary organisms that inhabit the water surface, forms a dense film that prevents light penetration. Thus, there is a reduction in the photosynthetic rate of plants located in the lower layers, causing dissolved oxygen deficit and consequently aerobic organisms present in the ecosystem cannot survive (HARDMAN, MCELDOWNEY, *et al.*, 1993). Thus, the number of decomposing agents, such as algae, also increases, which release toxins affecting the water quality and making it unsuitable for human consumption. The sources of nitrogen and phosphorus in domestic sewage mainly come from the use of detergents and chemicals for cleaning purposes and from feces and urine, as well as fertilizers used in the agricultural.

Therefore, it is essential to perform an effective removal of nutrients in wastewater treatment plants (WWTP) in order to preserve the quality of water resources. In Brazil, most of the WWTPs use biological processes to remove nutrients and organic matter from domestic and industrial wastewaters. The main technology employed is the activated sludge process, which uses the influent wastewater compounds (pollutants) as a substrate for the growth of a diverse microbial community. However, activated sludge systems

show some disadvantages, since the microorganisms group together to form a flocculent sludge with a relatively low settling velocity, requiring large secondary settler tanks to separate the biomass from the treated effluent and thus raising the installation area of the WWTP.

Considering the limited availability of space for the construction of large WWTPs in urban centers, and the increase in the production of wastewater by the population, it is necessary to establish new strategies for the treatment of wastewaters. The WWTPs should be more compact and efficient in removing both carbonaceous substances and nutrients. In this context, the aerobic granular sludge (AGS) technology has emerged as a promising alternative to meet these requirements.

AGS consists of microbial agglomerates of spherical morphology with high surface area, which allows high adsorption of substances present in the wastewater (MORGENROTH, SHERDEN, *et al.*, 1997, ADAV, LEE, *et al.*, 2008). Aerobic granules present excellent sedimentation properties, facilitating the separation between the biomass and the treated effluent. As they are preferably grown in sequencing batch reactors (SBR), there is no need for secondary settlers. Moreover, the removals of organic matter and nutrients take place in the same reactor, reducing costs and installation space, and making the treatment more versatile. Moreover, high compactness of AGS associated with SBR operation favor biomass retention. Consequently, AGS promotes higher pollutants removal than conventional activated sludge-based systems.

On the other hand, the intense development of synthetic compounds, mainly from the pharmaceutical industry, has resulted in an increase of pollutants present in the water (BOLONG, ISMAIL, *et al.*, 2009). Advances in analytical chemistry techniques have been able to determine low concentrations (in the order of μg L^{-1} and ng L^{-1}) of these pollutants, commonly known as micropollutants. Among the principal substances classified as micropollutants are: drugs, persistent organic pollutants (POPs) and endocrine disrupting chemicals (EDCs) (BILA, DEZOTTI, 2007, BOLONG, ISMAIL, *et al.*, 2009).

Endocrine disrupting chemicals are exogenous substances that affect the endocrine system of humans and animals in different ways. Studies report that, in humans, EDCs can result in pathologies such as breast, uterus and prostate cancer, development of polycystic ovaries, changes in thyroid glands, decreased male fertility, among others. In animals, however, it may lead to changes in reproduction, sterilization and induction

of female characteristics in male organisms, as in the case of fishes (SCHÄFER, WAITE, 2002).

Due to their high estrogenic potential, natural estrogens such as 17β-estradiol, estrone, and estriol as well as the synthetic estrogen 17α-ethinylestradiol, are considered the most dangerous EDCs. They induce changes in the endocrine system of organisms present in water even at low concentrations. The main sources of estrogens in natural water are non-treated or poorly treated domestic wastewaters, as these compounds are excreted daily by humans in urine and feces (D'ASCENZO, DI CORCIA, *et al.*, 2003).

Despite the importance of removing these compounds from wastewater to avoid their negative impact on living organisms, there is a lack of understanding about the mechanisms of biological degradation of EDCs and the removal of these substances by conventional biological processes are insufficient (TERNES, STUMPF, *et al.*, 1999). To solve this problem, it is necessary to develop wastewater treatment processes capable of promoting the removal of these micropollutants.

Previous studies have proposed some effective treatment approaches for EDC degradation, mainly advanced oxidation processes (AOPs) such as: ozonation (ESPLUGAS, BILA, *et al.*, 2007, LEE, Y., VON GUNTEN, 2010, LARCHER, DELBÈS, *et al.*, 2012), photocatalysis (BELGIORNO, RIZZO, *et al.*, 2007, BENOTTI, STANFORD, *et al.*, 2009, MA, ZHANG, *et al.*, 2015) and activated carbon adsorption (WESTERHOFF, YOON, *et al.*, 2005, SNYDER, ADHAM, *et al.*, 2007, REUNGOAT, ESCHER, *et al.*, 2012).

Biological processes have become the target of researches aiming at EDC removal, with activated sludge (TERNES, STUMPF, *et al.*, 1999) and moving bed biofilm reactor (MBBR) the most investigated processes (LUO, GUO, *et al.*, 2014, JIANG, NGO, *et al.*, 2018). Nevertheless, there are few studies addressing the application of AGS to remove micropollutants, which represents a challenge to be met in the present work. Despite this technology is highly studied because of its effectiveness on the removal of pollutants, such as carbonaceous matter and nutrients (nitrogen and phosphorus), the removal of EDCs using this process may offer a new alternative environmentally friendly and at lower costs when compared to other physicochemical strategies.

Despite the sustainability and cost-effective characteristics inherent to biological processes, oxidative processes can also be effectively applied for the removal of EDCs, either stand-alone and acting as a polishing step of biologically treated secondary

effluents. In this context, photocatalytic processes with immobilized catalyst consists of a very interesting alternative, since it avoids a post-filtration step to remove the catalytic material dispersed in the water.

Based on the above, one of the objectives of this thesis was to evaluate the aerobic granular sludge process for the removal of the EDCs 17β-estradiol and 17α-ethinylestradiol along with organic matter and nutrients abatement. The degradation of both hormones by advanced oxidation process in a membrane micro reactor was also addressed in this research.

The specific objectives of this work were:

- Evaluate the performance of an AGS-SBR reactor in the combined removal of organic matter, nitrogen and phosphorous from a simulated domestic wastewater;
- Evaluate the influence of the solids retention time (SRT) on the granular biomass stability and pollutants removal capability;
- Investigate specific bacterial functional groups involved in nitrogen and phosphorus removal by fluorescence in situ hybridization;
- Monitor the effects of EDCs addition to the AGS reactor, both on COD and nutrients removal and on the stability of the aerobic granules;
- Follow the conversions of COD, nitrogen and phosphorus along with the SBR cycles under anaerobic and aerobic/anoxic conditions;
- Evaluate the amount of EDCs removed either by biodegradation or adsorption on the biomass by assessing both liquid (effluent) and solid (sludge) samples;
- Assess the estrogenic activity of the samples collected in the effluent of the biologic reactor through YES assay;
- Investigate the performance of a membrane micro reactor in EDC degradation and evaluate the effect of combining different advanced oxidation processes (UV/H_2O_2, UV/H_2O_2, photocatalysis, $UV/H_2O_2/TiO_2$) on the micropollutants removal;
- Compare the effect of the dosage method of the oxidant agent (permeation through the membrane and direct injection) on the removal efficiency of EDCs;
- Study the influence of the aqueous matrix (synthetic or real wastewater) on the performance and conversion of EDCs using the best process configuration with optimized operating variables in the membrane micro reactor;

2. Fundamentals and Literature Overview

For a better understanding of the subjects addressed in this thesis, this chapter presents a review of the main concepts reported in the literature, mainly related to the biological and advanced oxidation wastewater treatment processes as well as the growing problem of contaminants of emerging concern, specifically the endocrine disrupting chemicals.

2.1. Initial Considerations in Wastewater Treatment

The increase in population and the accelerated industrial growth are factors that negatively affect the environment, causing pollution and deterioration of natural resources (JORDÃO, PESSOA, 2011). The main sources of water pollution are solid waste, rainwater, domestic and industrial wastewaters that are discharged into the receiving bodies. These sources present high concentrations of organic matter and nutrients that can be harmful and must be considered in wastewater treatment processes.

According to Bassin and Dezotti (2008), the main steps in wastewater treatment, corresponding to the degree of removal desired are:

- Preliminary treatment: it aims to remove coarse materials such as suspended solids and to prepare the influent for the next treatment step. Among the main pre-treatment processes are screening, grit removal and equalization for pH and/or flow adjustment.
- Primary treatment: the objective is to remove suspended solids which are still present in the wastewater after preliminary treatment. Among the main primary treatment processes are primary sedimentation, and coagulation/flocculation.
- Secondary treatment: it is the stage in which the biodegradation of organic matter by microbial metabolism occurs, and can be accompanied by the removal of nutrients such as nitrogen and phosphorus. In this treatment, biological processes play a crucial role. Examples of biological treatment processes include activated sludge systems, membrane bioreactors,

biofilm systems such as the moving bed biofilm reactor and submerged fixed-film reactor, among others.

- Tertiary treatment: also known as polishing step, it is intended to remove the remaining amounts of pollutants that were not completely removed in the previous steps and thus improve the quality of the effluent. Usually this step is used to remove micropollutants present in the biologically treated effluent as well as toxic substances, dyes, toxins that cause undesirable odors, etc. The main processes used in tertiary treatment are advanced oxidation processes, ozonation, reverse osmosis, adsorption, electrochemical processes, among others.

2.2. Biological Treatment of Wastewaters

Biological treatment of wastewater fundamentally depends on the action of microorganisms, which degrade the complex organic compounds present in the aqueous matrices to carry out their metabolic activities, producing simpler compounds such as mineral salts, CO_2, H_2O, NH_3, CH_4, H_2S, among others (JORDÃO, PESSOA, 2011). The degradation of pollutants is performed by diverse microbial communities, composed mainly of bacteria, protozoa, micrometazoans, fungi, and worms present in the sewage (VON SPERLING, 2005). In the presence or absence of oxygen, the process of biodegradation of carbonaceous matter can occur by aerobic or anaerobic pathways and may involve assimilation of other nutrients as nitrogen and phosphorus.

Approximately 80% of bacterial cells are composed of water and 20% of dry matter, of which 90% corresponds to organic matter and 10% to inorganic matter (VON SPERLING, 2005). The chemical composition of the biomass can be represented as $C_{60}H_{87}0_{23}N_{12}P$, depending on the conditions of the medium. In the absence of any of these nutrients, the microbial growth can be limited.

The microorganisms present in the sewage are usually grouped in the form of flocs, ensuring a more stable and resistant environment (ADAV, LEE, *et al.*, 2008). Adhesion between cells is favored by the excretion of polymeric substances, mostly proteins and polysaccharides. These substances are released when microorganisms are in the decay phase (endogenous phase). Under these conditions, there is a shortage of substrate and microorganisms metabolize their own cell material, leading to the

phenomenon known as cell disruption or lysis (ADAV, LEE, 2008, SHENG, YU, *et al.*, 2010).

Depending on the way microorganisms agglomerate themselves, biological treatment systems can be classified into two groups: fixed (attached) or suspended biomass (BASSIN, DEZOTTI, 2008). In fixed biomass systems, microorganisms remain immobile on supports where they grow to form biofilms. On the other hand, in suspended biomass systems, microbial communities form agglomerates that circulate freely through the reactor (JORDÃO, PESSOA, 2011). Figure 2.1 shows the main biological systems used in wastewater treatment.

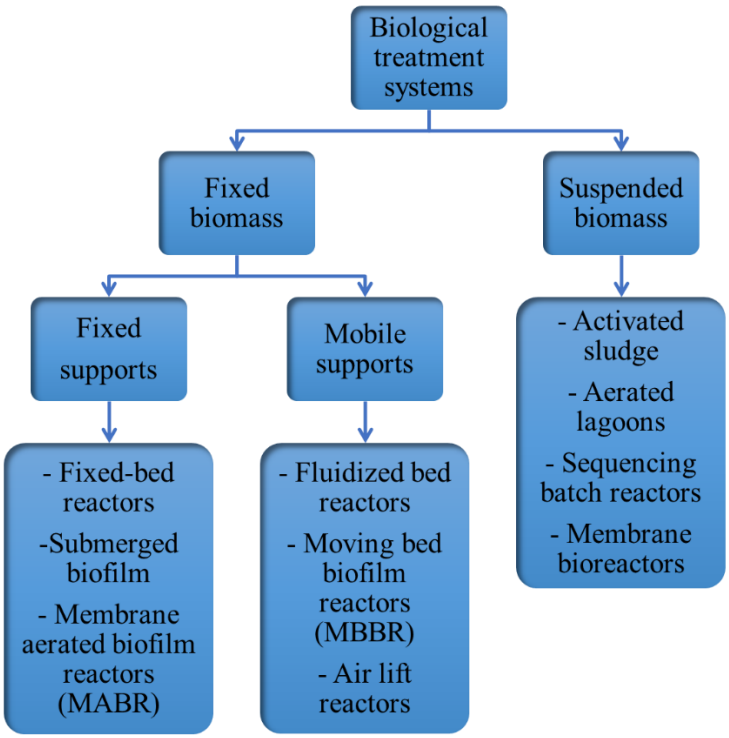


Figure 2.1 – Classification of biological treatment systems according to the biomass agglomeration. Source: Author.

Biological processes that use suspended biomass are mostly known due to activated sludge process, one of the first to be used in wastewater treatment plants. In the specific case of this work, a variant of the activated sludge process known was sequencing batch reactor (SBR) technology was used, and it will be explained in detail throughout this chapter.

2.2.1. Removal of Organic Matter

During the biological treatment of wastewater, interactions occur between the microorganisms and the substrate (pollutants), involving simultaneous or sequential chemical reactions of oxidation and reduction (VAN HAANDEL, VAN DER LUBBE, 2007).

Heterotrophic microorganisms use organic matter as a carbon source to carry out their metabolic processes. Depending on the degree of biodegradability, organic matter can be biodegradable (transformed into simple molecules) or recalcitrant (when it does not change its structure during the treatment) (BASSIN, DEZOTTI, 2008). Biodegradable organic matter is found in both soluble and particulate forms, where soluble is generally fast biodegraded and usually this form is preferably used by heterotrophic bacteria as substrate. Particulate organic matter, on the other hand, may present lower rates of biodegradation, requiring the action of extracellular enzymes to perform its hydrolysis (VON SPERLING, 2007).

In the biological treatment of wastewaters, there are essentially three types of processes that involve degradation of organic matter, depending on the environmental conditions and characteristics of the microorganisms: aerobic, anoxic and anaerobic.

The process of aerobic removal of organic matter is based on the oxidation of organic compounds in the presence of oxygen as the final electron acceptor. The main agents responsible for the degradation of organic matter are aerobic and facultative heterotrophic bacteria (VAN HAANDEL, VAN DER LUBBE, 2007), which use the oxygen present in the wastewater (dissolved oxygen), product of the photosynthetic activity of other microorganisms or even introduced by the mechanical devices or distributed through air diffusers. In general, the conversion process of carbonaceous matter under aerobic conditions can be expressed by Equation (2.1).

$$C_xH_yO_z + \frac{1}{4}(4x+y-2z)O_2 \rightarrow xCO_2 + \frac{y}{2}H_2O \quad (2.1)$$

When different types of electron acceptors are present in the medium, microorganisms use those that provide greater amounts of energy (VON SPERLING, 2007). For this reason, oxygen is primarily used in the oxidation reactions of organic matter and the process remains aerobic until it is completely depleted. At that moment, the cell respiration process can become anoxic, if nitrate is present in the medium, as it

would be the new electron acceptor used by microorganisms to supply their metabolic activities.

Otherwise, the anaerobic digestion process consists of the degradation of complex organic compounds in the absence of oxygen, which are transformed into biogas, a mixture of methane (60-70%) and carbon dioxide (30 - 40%), by the action of facultative or strictly anaerobic bacteria (BASSIN, DEZOTTI, 2008). Equation (2.2) represents a generic form of the conversion of organic matter under anaerobic conditions.

$$C_xH_yO_z + \frac{4x\text{-}y\text{-}2z}{4} H_2O \rightarrow \frac{4x\text{-}y+2z}{8} CO_2 + \frac{4x+y\text{-}2z}{8} CH_4 \quad (2.2)$$

The anaerobic digestion process is characterized by two main stages:

- Acidogenesis: represents the conversion of organic matter into organic acids by the action of acidogenic bacteria. At this stage, only the transformation of organic matter takes place, but there is no removal.
- Methanogenesis: conversion of organic acids into methane, carbon dioxide and water by methanogenic archaea. During this phase, there is removal of organic matter when methane is formed and transferred to the atmosphere.

Due to the complexity to characterize the large number of organic substances present in an environmental matrix, it becomes more practical to use variables that represent the amount of organic matter. Thus, there are two methods commonly used for the quantification of the organic material: biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

The BOD indicates the polluting potential of a wastewater. This method measures the amount of oxygen needed to biologically stabilize the organic matter present in a sample after a certain time (5 days) and at a certain temperature (20 °C) (FADINI, JARDIM, *et al.*, 2004). On the other hand, COD corresponds to the amount of oxygen needed to chemically oxidize the organic fraction of a given sample that is oxidizable by potassium dichromate in a strong acidic solution (DEZOTTI, 2008). The great advantage of COD is that this method is much faster compared to BOD, obtaining results in up to 2 hours, which makes this test more used for wastewater analysis. Consequently, the COD analysis allows to know the total oxygen demand and not only the biological demand.

Usually, COD ranges between 200 to 800 mg L^{-1} in domestic sewage, with an average value around 400 mg L^{-1} (JORDÃO, PESSOA, 2011).

2.2.2. Biological Removal of Nitrogen

Nitrogen is an important nutrient for all living organisms. In domestic wastewater, nitrogen is present in the organic form (amino acids, urea, uric acid and nitrogenous bases) and in the form of ammoniacal nitrogen (as free ammonia – NH_3 and as ionized ammonia – NH_4^+) (METCALF & EDDY, 2003). Organic nitrogen can be degraded to ammoniacal nitrogen by organic matter decomposing bacteria, in a process known as ammonification (VON SPERLING, 2007). Ammonification can occur during the primary or secondary treatment stages in WWTPs, where most compounds containing organic nitrogen are transformed into ammoniacal nitrogen. The relationship between free and ionized ammonia is given by Equation (2.3).

$$NH_3 + H^+ \leftrightarrow NH_4^+ \quad (2.3)$$

As the pH in the medium increases, the concentration of H^+ ions decreases, causing the reaction balance to shift to the left, favoring the formation of NH_3. At acid values of pH or close to neutrality, the reaction balance shifts to the right and, therefore, ammoniacal nitrogen is mostly in the NH_4^+ form (VON SPERLING, 2005). Ammoniacal nitrogen compounds favor the growth of algae in water bodies, so its elimination is necessary to avoid eutrophication problems (JORDÃO, PESSOA, 2011).

Normally, biological nitrogen removal processes involve different types of microorganisms and substrates. The first stage, known as nitrification, is characterized by the conversion of ammonium to nitrate under aerobic conditions, with oxygen as an electron acceptor. In the second stage, called denitrification, nitrate is converted to nitrogen gas under anoxic conditions, with nitrate being the electron acceptor (BASSIN, 2011). The main conversions taking place in the conventional biological nitrogen removal process are presented in Figure 2.2.

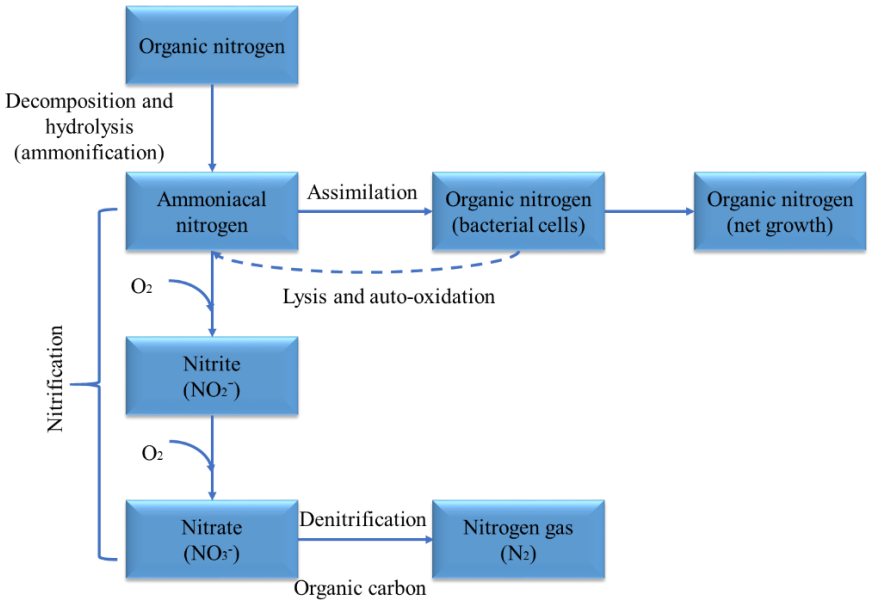


Figure 2.2 – Scheme of the overview biological nitrogen removal mechanisms. Adapted from Metcalf & Eddy (2003).

2.2.2.1. Nitrification

In the first stage of nitrification, known as nitritation, ammonia is transformed into nitrite by the activity of autotrophic ammonia-oxidizing bacteria (AOB), mainly from the genus *Nitrosomonas*. This type of bacteria obtain energy through the oxidation of an inorganic substrate, such as ammonia, by the action of specific enzymes as ammonia monooxygenase (AMO) (VON SPERLING, 2007, BASSIN, KLEEREBEZEM, *et al.*, 2012b). Equation (2.4) represents the global reaction of nitritation.

$$NH_3 + \frac{3}{2}O_2 \rightarrow NO_2^- + H^+ + H_2O \quad (2.4)$$

The second stage of the nitrification process is the nitratation, which consists in the conversion of nitrite to nitrate and occurs by the action of nitrite-oxidizing bacteria (NOB), mainly from genus *Nitrobacter* and *Nitrospira*, which use the enzyme oxidoreductase (YUAN, X., GAO, 2010, CYDZIK, WOJNOWSKA, 2011). The nitration reaction is shown in Equation (2.5).

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \quad (2.5)$$

When nitrification occurs, there is a strong demand of dissolved oxygen (DO) from the medium. It is used both by heterotrophic bacteria that perform organic matter removal and by nitrifying autotrophic bacteria, being a limiting factor in the metabolic processes of microorganisms (METCALF & EDDY, 2003).

Some environmental factors may influence the nitrification process, such as temperature, pH, dissolved oxygen concentration and substrate concentration. Table 2.1 list some favorable conditions for the nitrification process.

Table 2.1 – Favorable conditions for the nitrification process.

Parameter	**Optimum values for nitrification**	**Authors**
Temperature (ºC)	28 – 36	(METCALF & EDDY, 2003)
pH	7 – 8.5	(VAN HAANDEL, VAN DER LUBBE, 2007)
DO (mg O_2 L^{-1})	> 2	(NAKANO, IWASAWA, *et al.*, 2004)

In addition to the factors listed in Table 2.1, there is another important factor that affects the efficiency of the nitrification process: the solids retention time (SRT). As autotrophic bacteria present a slower growth rate than heterotrophic bacteria, a longer time is necessary to oxidize the ammonia. The higher SRT means a higher concentration of biomass in the biological reactor, therefore, a larger population of nitrifying bacteria (METCALF & EDDY, 2003). At the same time, there is a direct relationship between temperature and SRT in the nitrification process. Nitrifying bacteria are preferably mesophilic organisms, so once the temperature rises, their growth accelerate and the biomass concentration in the reactor increases. Thereby, it is possible to establish an indirect relationship between temperature and SRT, increasing the solids retention time in the system when there is a decrease in temperature to favor nitrification (GERARDI, 2002).

2.2.2.2. Denitrification

Denitrification is the complementary stage of nitrification, in which the nitrate formed as a product of ammonia oxidation is reduced to nitrogen gas, completing the process of biological nitrogen removal. Once the dissolved oxygen in the medium is

depleted, facultative anaerobic heterotrophic microorganisms (as *Pseudomonas, Achromobacter, Rhodopseudomonas, Halobacterium* and many others) use nitrate as a final electron acceptor to oxidize organic matter (BASSIN, KLEEREBEZEM, *et al.*, 2012b).

The denitrification process takes place in four sequential stages, in which specific enzymes are responsible for catalyzing the reactions (VAN KESSEL, SPETH, *et al.*, 2015). First, nitrate is reduced to nitrite, then nitrite is reduced to nitric oxide which is subsequently reduced to nitrous oxide and finally reduced to gaseous nitrogen. Equations (2.6) to (2.9) describe the denitrification process.

$$NO_3^- + 2\,e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (2.6)$$

$$NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O \quad (2.7)$$

$$2\,NO + 2\,e^- + 2H^+ \rightarrow N_2O + H_2O \quad (2.8)$$

$$N_2O + 2\,e^- + 2H^+ \rightarrow N_2 + H_2O \quad (2.9)$$

In comparison with nitrifying bacteria, heterotrophic organisms are less sensitive to environmental conditions, so once nitrification takes place, denitrification is also likely to occur if the required conditions are met. The absence of oxygen and the presence of organic matter in the medium are crucial factors for the denitrification process to occur (VAN HAANDEL, VAN DER LUBBE, 2007).

As observed in the denitrification reactions, the process of reducing nitrate to nitrogen gas consumes H^+ ions, and therefore, partially recover the alkalinity lost by nitrification. A range of pH values that favors denitrification rates are between 6.5 to 7.5 (VON SPERLING, 2007).

2.2.3. Biological Removal of Phosphorus

Phosphorus is an essential nutrient and exists in the environment both in organic and inorganic forms. Organic phosphorus is combined with organic matter in the form of proteins and amino acids, while inorganic phosphorus is present in the wastewaters mainly as orthophosphate and polyphosphate (JORDÃO, PESSOA, 2011).

The phosphorus biological removal from wastewater can be carried out by the Enhanced Biological Phosphorus Removal (EBPR) process. This process relies on the microbial communities to incorporate phosphorus into the cells and perform the removal of this nutrient (BARNARD, 1975). The microorganisms used for this purpose are heterotrophic bacteria known as Polyphosphate Accumulating Organisms (PAOs), which are selected by subjecting the sludge to alternating anaerobic to aerobic conditions.

Under anaerobic conditions, fermentative bacteria degrade the organic matter, mainly acetate and propionate, into volatile fatty acids (VFA). VFA is a substrate for PAOs that polymerize and store them as polyhydroxyalkanoates (PHA) (VAN LOOSDRECHT, HOOIJMANS, *et al.*, 1997). Depending on the carbon source used, different types of PHA are produced, such as polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), and polyhydroxy-2-methylbutyrate (PH2MB). The most common PHA is PHB, which is formed from acetate degradation (MINO, VAN LOOSDRECHT, *et al.*, 1998). For the conversion of VFA to PHA, cells need energy, which is obtained by breaking the bonds of intracellular polyphosphate molecules (Poly-P), which is accompanied by a release of phosphate into the liquid medium.

During the aerobic or anoxic phase, when the organic substrate depletes, PAOs use PHA stored as substrate and energy source to perform their growth, cell maintenance, glycogen synthesis and phosphate absorption present in the medium to reestablish the polyphosphate pools inside the cells (WENTZEL, LOTTER, *et al.*, 1991). Due to the rapid bacterial growth over this phase, the phosphate absorption is higher than the phosphate release during the anaerobic phase and consequently there is an effective removal of this nutrient from the medium. The detailed biological phosphorus removal mechanisms are represented in Figure 2.3.

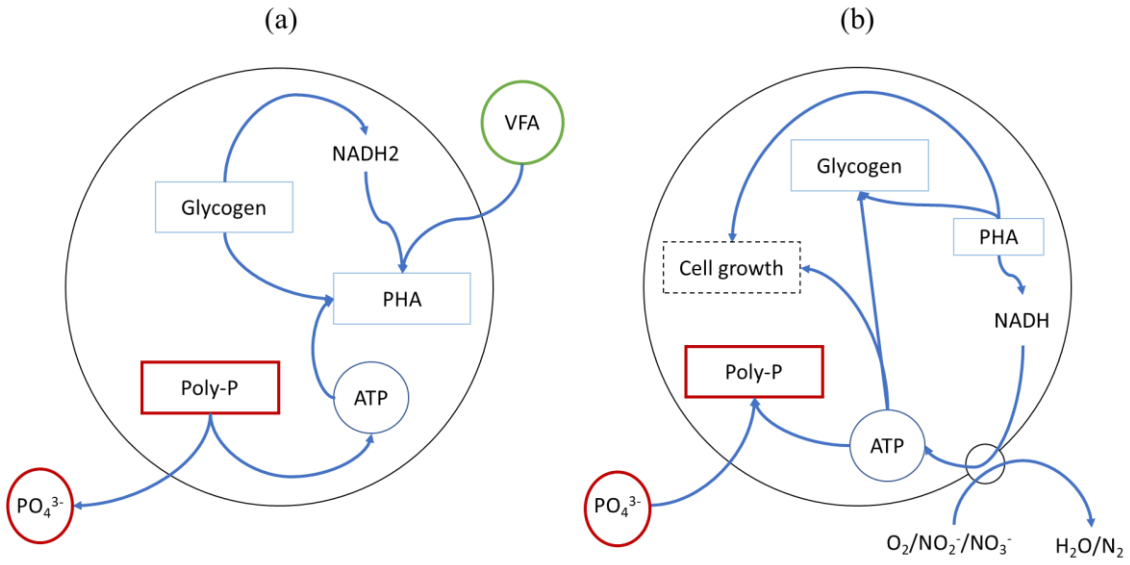


Figure 2.3 – Metabolism of polyphosphate accumulating organisms during the biological phosphorus removal process under: (a) anaerobic and (b) aerobic or anoxic conditions. Adapted from Wentzel, Lotter *et al.* (1991).

The operating conditions of the reactor influence the selection of PAOs compared to other heterotrophic microorganisms (BASSIN, KLEEREBEZEM, *et al.*, 2012b). Efficient phosphorus removal is achieved when most of the available organic matter is consumed during the anaerobic period. Therefore, depending on the influent wastewater composition, it is necessary to extend or decrease the duration of the anaerobic phase to allow complete biodegradation of the carbonaceous matter.

However, there are other type of microorganisms that compete with PAOs for the available organic matter under anaerobic conditions, being known as Glycogen Accumulating Organisms (GAO) (OEHMEN, SAUNDERS, *et al.*, 2006). The metabolism of GAOs is similar to PAOs, but they do not store or release polyphosphate. Consequently, GAOs do not contribute to the phosphorus removal from wastewater and therefore they are considered as undesirable in EBPR systems.

Several environmental factors, such as temperature, pH and DO concentration, may affect the biological phosphorus removal process (METCALF & EDDY, 2003). Some studies report that at low temperatures (around 10 – 20 ºC), phosphorus removal rates are improved. This is because at lower temperatures the growth of GAOs decreases,

favoring the establishment of PAOs in the reactor (LOPEZ-VAZQUEZ, OEHMEN, *et al.*, 2009).

Another important parameter in phosphorus removal processes is the SRT. Due to the high growth of cells in the reactor, especially during the aerobic phase, it is important to remove the excess sludge periodically to guarantee an efficient phosphorus removal previously stored in biomass. Lee, Kim and Chung (2007) tested the efficiency of phosphorus removal by alternating the SRT between 15 and 30 days. They found that the highest removal rates (around 93%) were obtained at the SRT of 20 days. On the other hand, Li et al. (2016) found that the SRT can be maintained up to 30 days reaching high phosphorus removal efficiencies (92%). On the other hand, a high SRT generates a low efficiency of phosphorus removal due to a saturation of this compound inside the PAOs causing a maximum removal to be achieved.

2.3. Biological Systems for the Simultaneous Removal of Nutrients

The main problem faced by the conventional activated sludge system is its inability to completely remove nutrients (N and P) from the sewage, being mostly used to remove organic matter and, with some modifications, removal of ammoniacal nitrogen can achieved by nitrification (GONZALEZ-MARTINEZ, MUÑOZ-PALAZON, *et al.*, 2017). In this context, new technologies aiming at the combined removal of organic matter and nutrients were developed and implemented. Among the most used systems in the industry are: Bardenpho system, University of Cape Town (UCT) system and the sequencing batch reactor (SBR) (BASSIN, 2011). This section addresses generic aspects focusing on the SBR system, which was used for the development of this thesis.

2.3.1. Sequencing Batch Reactor (SBR)

The sequencing batch reactor is a biological wastewater treatment system that consists of a single tank operating in an intermittent flow where the organic matter oxidation, nutrients removal and biomass/effluent separation happen. According to Von Sperling (2005), by using a single unit, the processes associated with the treatment of wastewaters become sequences in time, and not different compartments as in the conventional activated sludge process. All steps occur in a complete mixing reactor using operating cycles with a defined duration.

Due to the high level of system automation, which combines level sensors, timers and microprocessors, the process becomes very precise and flexible, allowing favorable conditions for the nitrification, denitrification and biological phosphorus removal processes through aerobic, anaerobic or anoxic phases (WAGNER, J., GUIMARÃES, *et al.*, 2015).

In general, the reactor consists of a variable volume tank, where the total volume (V_T) is composed of two independent fractions: the stationary volume (V_0) and the filling volume (V_F). The stationary volume comprises the volume occupied by the settled biomass (V_S) and the remaining volume (V_R) is the volume of treated effluent that remains inside the reactor. A typical column-type SBR and its characteristic volumes are represented in Figure 2.4.

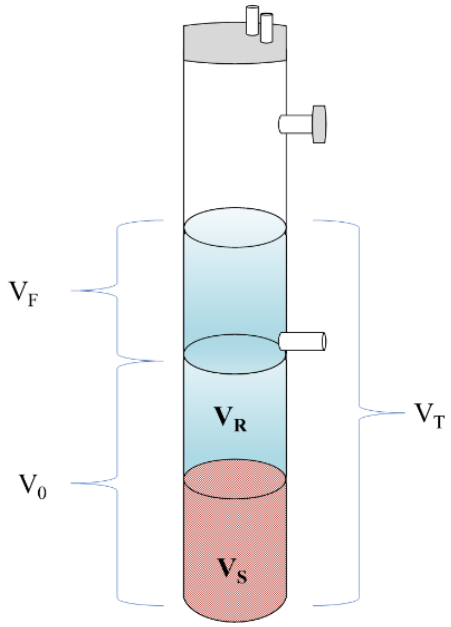


Figure 2.4 – Schematic representation of a sequencing batch reactor indicating the different volumes occupied by the liquid and biomass. Source: Author.

The SBR operation usually involves four sequential phases: influent feeding, aerated phase, biomass settling and effluent withdrawal, which are presented in Figure 2.5. The successive occurrence of all these phases composes a typical cycle, and throughout the day, the bioreactor can be subjected to several cycles (JORDÃO, PESSOA, 2011). Below, a brief description of each one of the phases:

- **Feeding phase:** corresponds to the influent filling into the reactor. In this phase, the air diffusers can be turned on or off, promoting aerobic, anoxic or anaerobic conditions. This last type of filling promotes the growth of

slow-growing heterotrophic organisms and prevents the growth of filamentous organisms in the reactor (FIGUEROA, VAL DEL RIO, *et al.*, 2015), while allowing the release of orthophosphates by PAOs. Therefore, it is often used when biological phosphorus removal is targeted. It is important to highlight that this operational phase can be operated in such a way that most of the substrate is assimilated by microorganisms. For this purpose, the contact time between the influent and the settled biomass bed has to be enough to favor the conversion of organic matter.

- **Aerated phase:** the air pumping devices are turned on and air is distributed by porous diffusers throughout this phase, allowing reactor homogenization and close contact between the wastewater and the microorganisms. The latter continue to degrade the organic matter that was not completely removed during the feeding phase and nitrification and phosphorus absorption into the cells may occur. According to Al-Rekabi, Qiang and Qiang (2007), the aeration phase is the longest, and can last up to 50% or more of the total cycle time.
- **Settling phase:** over this phase, the air diffusers are turned off and the suspended biomass settles down to the bottom of the tank, occupying a certain volume (V_S). In this step, the sludge is effectively separated from the treated effluent. If the solids do not settle quickly, they can be removed from the reactor during the withdrawal phase, a phenomenon known as biomass washout, deteriorating the quality of the treated effluent (WAGNER, J., GUIMARÃES, *et al.*, 2015). This problem can be solved by increasing the settling time as a function of the solids settling velocity (LIU, Y. Q., TAY, 2015).
- **Withdrawal phase:** the treated effluent is removed from the reactor. In this phase, 25 to 50% of the liquid is drained is such a way that the settled biomass is not disturbed.

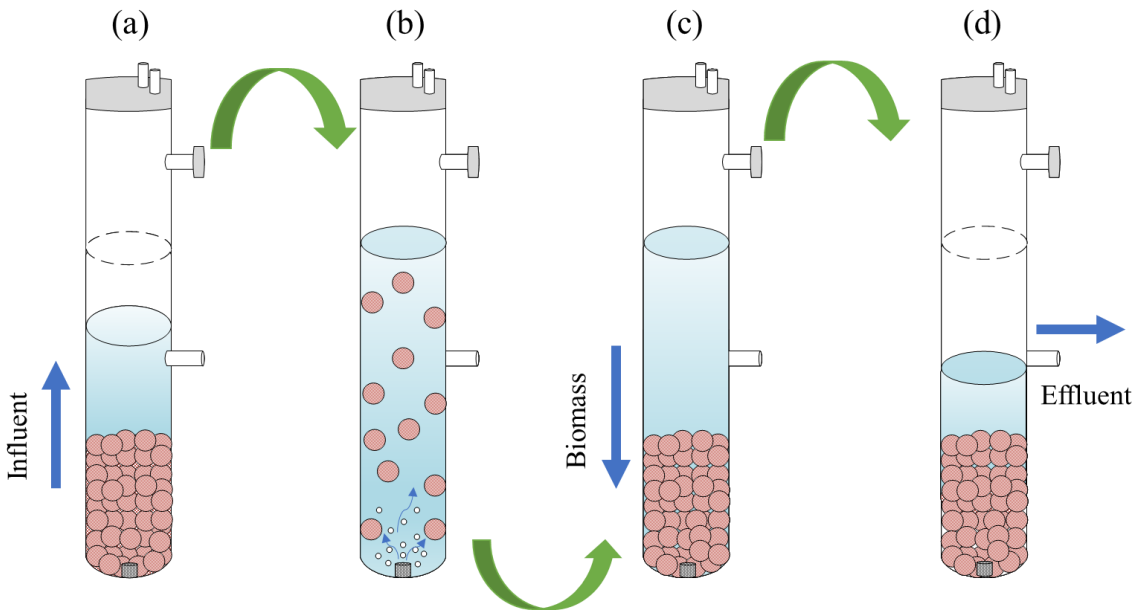


Figure 2.5 – Operating phases throughout a typical SBR cycle: (a) feeding, (b) aeration, (c) settling and (d) withdrawal. Source: Author.

Sequencing batch reactors have become a very attractive alternative for wastewater treatment, especially where space is limited. This system occupies a smaller installation area than a conventional activated sludge technology. Depending on the desired treatment level, according to the treated effluent quality requirements, modifications can be made in the operating cycles, such as the insertion of an anoxic phase or intermittent feeding in order to improve, for example, the denitrification process (VON SPERLING, 2007). The choice of incorporating changes along the phases or periods in each cycle gives flexibility to the process, which brings advantages such as the ease adaptation to fluctuations in the organic loads and the possibility of achieving complete nutrient removal (AL-REKABI, QIANG, *et al.*, 2007).

2.4. Aerobic Granular Sludge

The granular sludge technology emerged in the 1980s and was initially associated with anaerobic processes in upflow anaerobic sludge blanket reactors (UASB) (LETTINGA, VAN VELSEN, *et al.*, 1980). Due to the long reactor start-up, the requirement of high temperatures and the low efficiency of nitrogen and phosphorus removal by the anaerobic granular sludge, the possibility of developing aerobic granules was considered (ADAV, LEE, *et al.*, 2008).

In the late 1990s, Morgenroth et al. (1997) reported the formation and application of aerobic granular sludge (AGS) using an SBR. Since then, numerous researches have been carried out in order to understand the granulation process and to obtain a better performance in the organic matter and nutrients removal from wastewaters.

The first patent for the AGS process was granted to Mark Van Loosdrecht and Sef Heijnen in 1998. In 2005, a partnership between the Technological University of Delft, The Netherlands, together with the engineering consulting company Royal Haskoning DHV, patented the technology to implement it in industrial applications under the name of Nereda®. Currently, this company has built WWTPs based on AGS in 10 countries around the world, including Brazil, where 6 plants are operating or under construction in the states of São Paulo, Rio de Janeiro, Tocantins and Pernambuco.

Aerobic granules are compact microbial agglomerates with a well-defined appearance (DE KREUK, DE BRUIN, 2004). In comparison with the biological flocs in the activated sludge process, aerobic granules are more resistant and have dense structures. Aerobic granules allow high biomass retention to be reached in the reactor and show excellent settling capacity, thus an effective separation of the treated effluent can be obtained without the need of additional settling tanks (MORGENROTH, SHERDEN, *et al.*, 1997, DE KREUK, DE BRUIN, 2004, ADAV, LEE, *et al.*, 2008, ZHANG, Q., HU, *et al.*, 2016). Thanks to these advantages, the reactors operating with aerobic granular sludge promote high organic matter and nutrient removal from waste streams (LIU, X. W., YU, *et al.*, 2009).

Aerobic granules are generally grown in SBR systems, under temporal operating cycles between 3 and 6 h, where their formation and growth is favored (BEUN, HENDRIKS, *et al.*, 1999). According to De Kreuk, Pronk and Van Loosdrecht (2005), to be considered granular sludge, the aggregates must have a diameter greater than 0.2 mm and the amount of granules must correspond to at least 80% of the volatile suspended solids in the reactor.

Due to their high surface area, porosity and compact structure, aerobic granules also play an important role in the adsorption of toxic compounds. Xu and Liu (2008) proposed that some mechanisms, such as ion exchange, bonds to extracellular polymers and chemical precipitation, benefit the removal of substances such as Cd^{2+}, Cu^{2+} and Ni^{2+}. Moreover, Guibaud et al. (2009) attributed the removal efficiency of compounds such as Cd^{2+} and Pb^{2+} to the high chemical affinity between these elements and the polymeric substances present on the aerobic granules surface.

However, a major challenge is the maintenance and structural stability of the aerobic granules, which depend on several factors such as environmental conditions, air flow rates, applied organic loads and reactor operating conditions, among others (YUAN, S., GAO, *et al.*, 2017).

2.4.1. Formation of Aerobic Granules

The aerobic granulation process is complex and involves a series of mechanisms and conditions necessary for achieving stable granules. Several hypotheses have been proposed in different studies to explain the phenomena involved in the granulation process, but there is still no definitive theory.

At the beginning of the research on aerobic granular sludge, Beun et al. (1999) proposed that in the first stage of granulation, fungi, which are the dominant population in the system, form mycelia with good settling properties and could be easily retained inside the bioreactor. Due to the applied shear forces, fungi form filamentary aggregates with diameters ranging 5 to 6 mm, which would serve as an immobilization matrix for all bacterial communities present in the reactor. Thus, the bacteria would grow adhered and form colonies being able to stay inside the reactor. However, the role of fungi was not confirmed in later studies in the field.

Liu and Tay (2002) described the aerobic granulation process as an integration of the following steps:

- Contact between microorganisms due to hydrodynamic, thermodynamic, diffusive and gravitational forces.
- Stabilization of cellular aggregates thanks to physical (Van der Waals), chemical (ionic attractions and hydrogen bonds), biochemical (cell surface dehydration) and thermodynamic (free energy and surface tension) forces.
- Production of extracellular polymeric substances (EPS) that facilitate the adhesion between the cells and helps the new cells grow attached to each other.

The use of techniques such as scanning electron microscopy (SEM) and optical microscopy, have helped researchers as Weber et al. (2007) to confirm these hypotheses. It was discovered that the granules are composed mainly of bacteria, EPS, fungi and

protozoa. In their study, it was found that mature granules are composed by several types of bacteria organized in different layers where nitrification, denitrification and other processes occur.

Recent studies confirm the granulation mechanism proposed by Liu and Tay (2002) and propose additional details as important aspects to be considered during the aerobic granules formation process (HU, ZHANG, *et al.*, 2016, WILÉN, LIÉBANA, *et al.*, 2018, BASSIN, TAVARES, *et al.*, 2019). A contribution by Wang et al. (2017) provides information on the Quorum Sensing mechanism and the importance of cells transmitting information on the granules formation. This process can accelerate the EPS production facilitating the microbial adhesion. Figure 2.6 shows the different stages along the granulation process.

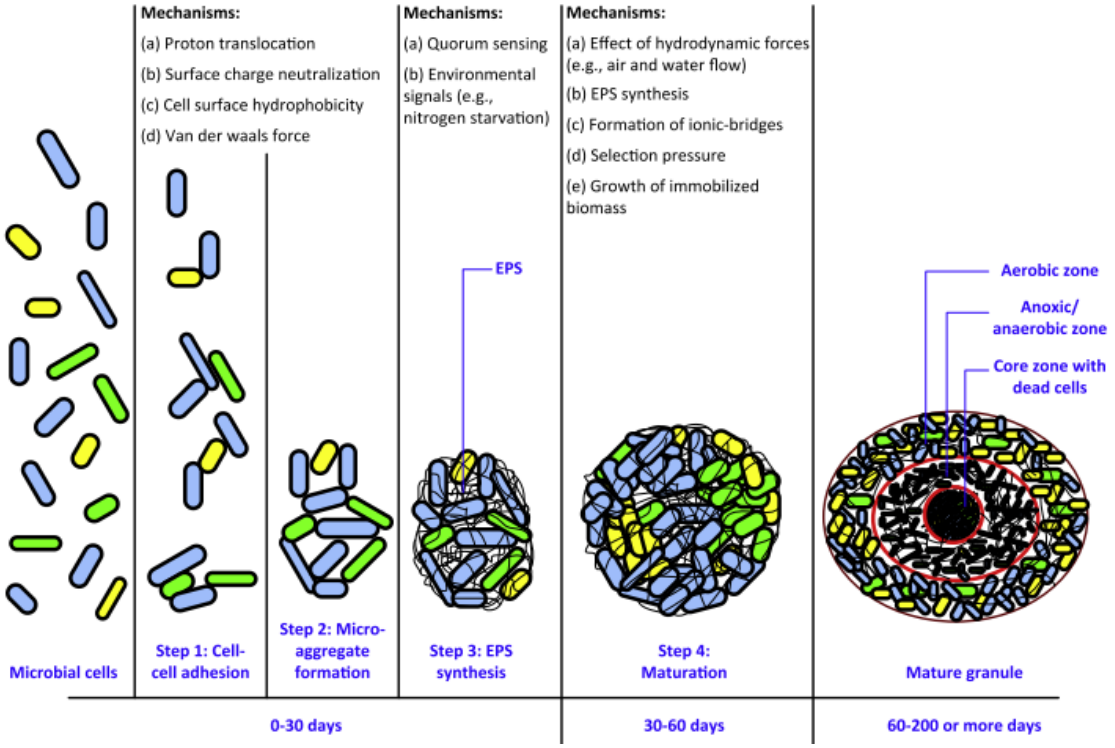


Figure 2.6 – Aerobic granular sludge formation and different mechanisms over the granulation stages (SARMA, TAY, *et al.*, 2017).

2.4.2. Factors Affecting Granulation

The formation of aerobic granules is a process that can be influenced by different operational conditions, including the type of substrate, applied organic load, hydrodynamic shear forces, hydraulic retention time (HRT), settling time, reactor

configuration, inoculum origin and environmental factors such as pH, temperature and dissolved oxygen (DO) concentration (MOY, TAY, *et al.*, 2002, DE KREUK, PRONK, *et al.*, 2005, DE KREUK, VAN LOOSDRECHT, 2006).

One of the key parameters for the AGS formation is the settling velocity. According to Wang et al. (2007), the increase in the selective pressure created by decreasing the settling time and increasing the volume exchange ratio in a SBR favors the granulation process. Under these conditions, low settling times (between 2 to 20 min) aid the washout the flocculent biomass with low settling velocities, while preserving the higher agglomerates that settle faster (BEUN, HENDRIKS, *et al.*, 1999).

The settling velocity, the density and the resistance of granules are associated to the shear forces applied in the system (LIU, Y., TAY, 2002). High shear force is reached by the high aeration rate supplied, which stimulates the respiration of aerobic microorganisms on the granules surface, accelerating the production of EPS. The release of EPS increases the hydrophobicity of cells and, as a consequence, their adhering potential (ADAV, LEE, 2008). In addition to increasing the EPS production, the high aeration rates applied scrape the granule surface, generating a smooth surface that improves its structural integrity and provides enough oxygen to perform the substrate degradation (SHOW, LEE, *et al.*, 2012). In the literature, some authors report favorable air velocities for the granulation process between 0.025 to 0.041 m s^{-1} (LIU, Y., TAY, 2002, LIU, X. W., YU, *et al.*, 2009).

On the other hand, the granules density depends on the microbial growth rate, with high growth rates leading to less dense granules (DE KREUK, PRONK, *et al.*, 2005). This fact is because high biomass growth rates stimulate the proliferation of filamentous microorganisms, which cause the granule density to decrease and, consequently, its settling velocity also decreases in a phenomena called *bulking sludge* (MORGENROTH, SHERDEN, *et al.*, 1997).

Some authors attribute these high microbial growth rates to the type of substrate consumed during the feeding phase. Tay, Liu and Liu (2001) cultivated granules using glucose and acetate as carbon sources in separate reactors and found that there was a predominance in the formation of filamentous bacteria in the reactors fed with glucose as a carbon source. Moy et al. (2002) attributed this to the fact that easy biodegradable substrates may accelerate the growth of heterotrophic microorganisms, while more complex substrates such as acetate, retard the growth of filamentous microorganisms in the granules. This hypothesis was confirmed by Zhou et al. (2014), who obtained similar

results. Also, Wan et al. (2015) reported that when replacing acetate with propionate as a substrate, the formed granules showed absence of filamentous microorganisms, being more dense and compact.

Environmental factors such as pH and temperature also influence microbial growth and, therefore, play a crucial role in the granulation process. Low pH values contribute to the proliferation of fungi, which may be important in the beginning of granulation, as stated in pioneering studies on aerobic granulation (BEUN, HENDRIKS, *et al.*, 1999). Nevertheless, the overgrowth of these microorganisms can generate large granules with DO diffusion problems and, consequently, fragile and unstable biomass (LIU, Y., TAY, 2004). Studies by Yang, Li and Yu (2008) and Wan et al. (2014) pointed that using alkaline media (pH = 8), the granulation process was favored with predominance of bacteria and absence of fungi resulting in more compact and stable granules.

The temperature effect on the formation of stable granules has also been studied. Several authors have managed to grow aerobic granules at temperatures between 20 - 30 ºC (DE KREUK, PRONK, *et al.*, 2005, LOPEZ-VAZQUEZ, HOOIJMANS, *et al.*, 2009, SONG, Z., REN, *et al.*, 2009, BASSIN, TAVARES, *et al.*, 2019). Research by De Kreuk, Pronk and Van Loosdrecht (2005) showed that it is possible to form granules at low temperatures (8 ºC) but they were not stable, with overgrowth of filamentous organisms. High temperatures were tested by Song et al. (2009), who report that it is possible to form aerobic granules up to 35 ºC, but the removal of nutrients decreases as the temperature increases.

According to Strevett and Chen (2003), when microorganisms are subjected to periods with lack of substrate, they increase their hydrophobicity due to the scarcity of carbonaceous material, facilitating their agglomeration. Nonetheless, long periods of starvation decrease the granules stability, leading to their disintegration (YUAN, GAO, *et al.*, 2017). For this reason, the way how the reactor is fed is important to achieve stable granules. Generally, in sequencing batch reactors, two characteristic periods are identified in the presence (*feast*) and absence (*famine*) of substrate. At the beginning of the anaerobic phase, the available substrate is maximum, being consumed quickly by the microorganisms until reaching a minimum value where it is missing (DE KREUK, VAN LOOSDRECHT, 2004). This alternation between availability and absence of substrate leads to the formation of more stable and defined granules.

Another important factor in the granulation process is the DO concentration, since this is an essential parameter that controls the microbial metabolism and hence, the cell growth. Some authors have reported DO concentrations above 2 mg L^{-1} as favorable for the granules formation (BEUN, HENDRIKS, *et al.*, 1999, LIU, Y., TAY, 2002, DE KREUK, VAN LOOSDRECHT, 2004). However, energy consumption increases due to the high intensity of air that must be supplied to the system. De Kreuk and Van Loosdrecht (2004) suggested alternating the reactor conditions in aerobic and anaerobic (or anoxic) to reduce operating costs without harming the granulation process. The research carried out by Wan, Bessière and Spérandio (2009) showed that alternating an anoxic feeding phase with an aerobic phase with no substrate fed resulted in granules with good structural properties.

The organic load applied have shown to influence the aerobic granules formation. Beun et al. (1999) stated that it was possible to form granules with an influent organic load between 2.5 and 7.5 kg COD m^{-3} d^{-1}. Nevertheless, Moy et al. (2002) managed to grow granules by expanding the organic load range between 2.5 and 15 kg COD m^{-3} d^{-1}. On the other hand, Liu and Tay (2002) found that there was a difficulty in forming aerobic granules when the applied organic loads were less than 2 kg COD m^{-3} d^{-1}. Other authors found no significant effect of the supplied substrate concentration, nevertheless, they reported that the granules physical and structural characteristics may be compromised. In the study published by Liu, Tay and Liu (2003), the authors observed that when increasing the organic load from 3 to 9 COD m^{-3} d^{-1}, the granules increased their diameter from 1.3 to 1.9 mm, becoming less resistant. This fact was attributed to the high available substrate concentration that promotes the formation of filamentous microorganisms who affect the granules structural properties (LIU, Y., LIU, 2006).

In the specific case of SBR systems, it is possible to control the substrate concentration by adjusting the hydraulic load that depends on the HRT and the volume exchange ratio (V_F V_T^{-1}). When the reactor is fed with a low organic matter concentration, as in the case of domestic wastewater, the hydraulic load applied must be high to favor the granulation process. Beun et al. (1999) and Liu et al. (2009) suggest high volume exchange ratio (around 0.50 - 0.75) and low HRT (around 3 h) in order to obtain a high hydraulic load favorable for the granules development.

Overall, the aerobic granulation process is affected by numerous factors that act together, making it a complex process which needs constant research. Although several aspects related to the granules formation process and the factors that may affect this

process have been studied, there are still gaps regarding the maintenance and stability of the granules in the long-term, which represent a challenge for this technology.

2.4.3. Simultaneous removal of nutrients by aerobic granules

Aerobic granules present a heterogeneous structure, where different layers can be identified. Normally, their surface is smooth, dense and strong. A high microbial density is identified in the outer layers, while a low biomass density is found in the inner layers as a result of oxygen limitation (TAY, LIU, *et al.*, 2001). Consequently, oxygen and substrate gradients are created between the surface and the inner part of the granule, emerging anoxic and anaerobic zones inside, favoring the coexistence of nitrifying, denitrifying and phosphate accumulating microorganisms in the same environment (DE KREUK, VAN LOOSDRECHT, 2004). This stratification with different microbial communities within the granule allows simultaneous removal of organic matter and nutrients (BASSIN, KLEEREBEZEM, *et al.*, 2012a).

According to Winkler et al. (2013), the microorganisms responsible for the oxidation of organic matter, as well as nitrifying bacteria, are found mainly on the granules surface, where there is higher availability of oxygen, while in the inner layers (anoxic and anaerobic), denitrifying bacteria are distributed along with PAOs and GAOs. A schematic representation of the internal structure of the granule and the distribution of microorganisms in the different layers is shown in Figure 2.7.

The organic matter and nutrients underlying mechanisms in AGS are similar to those found in the activated sludge systems, with the difference that in aerobic granules the biological processes take place in the multiple layers of these microbial agglomerates throughout the SBR cycle (DE KREUK, VAN LOOSDRECHT, 2006).

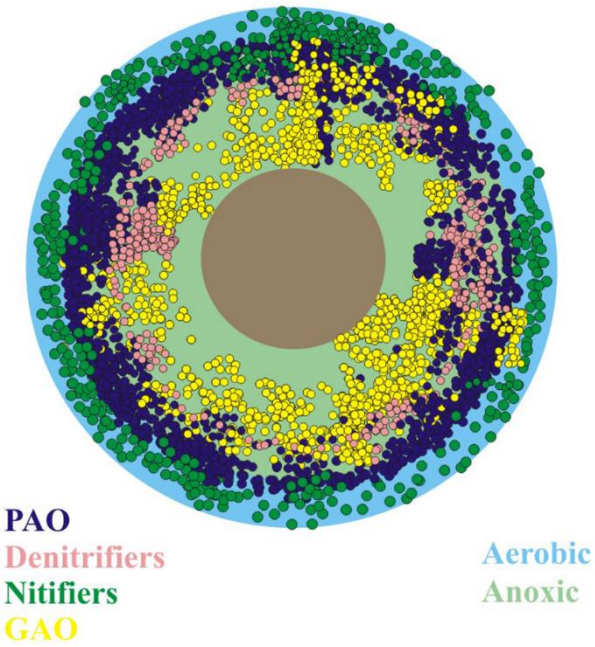


Figure 2.7 – Internal structure and microbial distribution in aerobic granular sludge. Adapted from Winkler, Kleerebezem *et al.* (2013).

During the feeding phase, the substrate concentration in the system is high (*feast*) and is readily available for heterotrophic bacteria on the granules surface. Over the aerated phase, part of the substrate is oxidized to CO_2 and the rest is stored in the form of PHA. Intracellular polymer storage can be intensified if a prolonged feeding phase without external electron acceptors (anaerobic conditions) is implemented (MORGENROTH, SHERDEN, *et al.*, 1997). Over the aerated phase, DO is quickly consumed by the aerobic microorganisms which carry out the conversion processes of remaining organic matter, storage inside cells, nitrification and cell growth (DE KREUK, PRONK, *et al.*, 2005).

The nitrification products (nitrite and nitrate) diffuse into the granule, where denitrifying bacteria use PHA as a carbon source to carry out the denitrification process. As a result, nitrogen removal occurs inside the granule by simultaneous nitrification and denitrification processes (SND) (DE KREUK, DE BRUIN, 2004). As the concentration substrate decreases (*famine*), the DO concentration in the liquid increases, because the respiration rate of heterotrophic microorganisms is low, and oxygen is able to penetrate more easily inside the granules affecting the SND process efficiency (NAKANO, IWASAWA, *et al.*, 2004).

It is important to note that during the anaerobic feeding phase, it is also possible that denitrification of NOx compounds remained from the previous cycle to occur, using

the fed substrate as a carbon source (BASSIN, PRONK, *et al.*, 2011). On the other hand, feeding under anaerobic conditions favors the growth of PAOs in the reactor. According to De Kreuk, Heijnen and Van Loosdrecht (2005), by alternating an anaerobic feeding phase with an aerobic reaction phase, it is possible to enrich the granular sludge with PAO, obtaining good phosphorus removal. During the feeding phase, PAOs and GAOs store the substrate in the form of PHA, and PAOs release phosphate into the medium as part of the EBPR process (BASSIN, KLEEREBEZEM, *et al.*, 2012b).

During the aeration stage, in the aerobic/anoxic regions of the granule, PAOs and GAOs oxidize the PHA previously stored with oxygen or nitrate/nitrate as electron acceptor, while PAOs use the energy to store phosphate inside the cells as poly-P with a consequent decrease in the phosphate concentration in the liquid medium (LOPEZ-VAZQUEZ, HOOIJMANS, *et al.*, 2009). Figure 2.8 displays the different nutrient removal mechanisms inside the aerobic granules in an aerated environment.

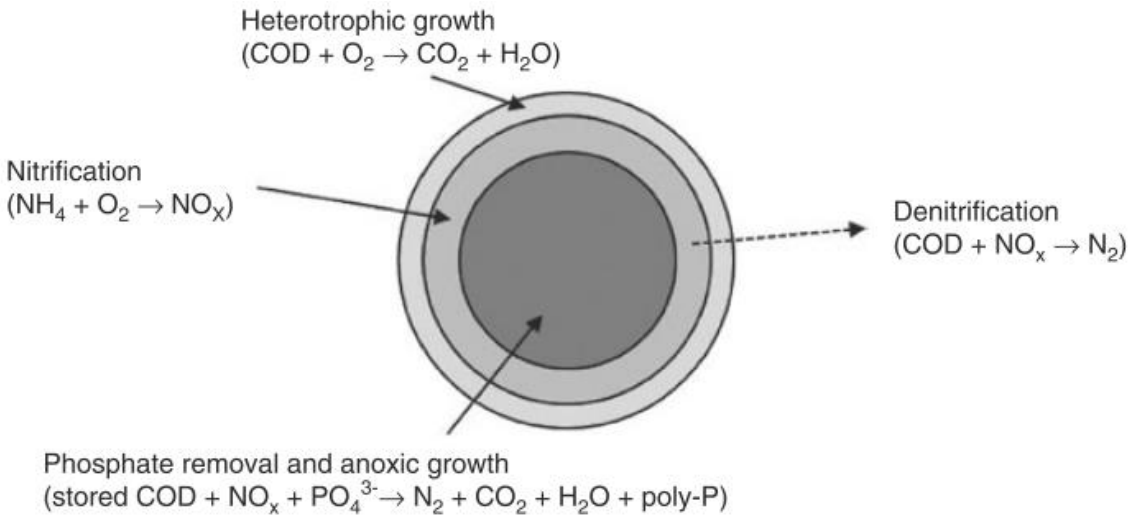


Figure 2.8 – Nutrient removal processes in the different layers of aerobic granules under aeration (BASSIN, 2018).

2.5. Endocrine Disrupting Chemicals (EDCs)

Compounds known as endocrine disrupters or endocrine disrupting chemicals are a group of substances that interfere with the functioning of the endocrine system. The latter consists of a series of glands that secrete hormones of vital importance for the growth, development and functioning of the organism in animals and humans.

According to the European Commission (1999), EDCs can affect the endocrine system in at least three ways:

- Mimicking the action of hormones and thus, triggering similar chemical reactions in the body;
- Blocking receptors in cells that receive hormones (hormone receptors), preventing the action of natural hormones;
- Affecting the synthesis, transport, metabolism and excretion of natural hormones, changing their concentrations.

A large amount of substances can affect the endocrine system such as: alkylphenols, phthalates, pesticides, polychlorinated biphenyls, bisphenol A, drugs and estrogens (BILA, DEZOTTI, 2007). The estrogens, both natural and synthetic, have been classified as the major contributors to endocrine disruption in organisms present in aquatic environments, since their high estrogenic potential is able to cause negative effects even at very low concentrations (ROUTLEDGE, SHEAHAN, *et al.*, 1998).

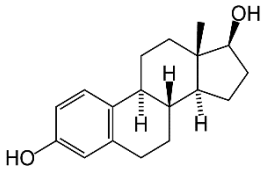
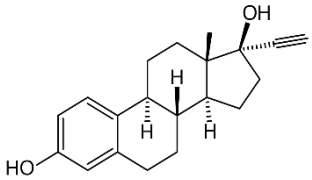
Estrogens are steroid hormones, synthesized from cholesterol that have a common molecular structure composed of a phenolic ring, two cyclohexane rings and a cyclopentane ring. The phenolic ring is responsible for the estrogenic activity of this type of compounds (YU, DEEB, *et al.*, 2013). The classification of estrogens can be performed in two groups: natural, when they are spontaneously produced by the sexual organs and by the adrenal glands, or synthetic. Natural estrogens are responsible for the development of secondary female characteristics that appear at the beginning of puberty, as well as the growth of mammary glands during the gestation period, and also stimulate heat in animals (BILA, MONTALVÃO, *et al.*, 2007). On the other hand, synthetic estrogens are produced mainly by the pharmaceutical industries, with the purpose of interfering and modulating the endocrine system, being commonly used in birth control pills, in hormone replacement therapies, or to treat diseases such as prostate, breast, and endometrial cancer, among others.

Due to their high estrogenic potency and for the considerable amounts that are constantly released into the sewers, the natural estrogens estrone (E1), 17β-estradiol (E2) and estriol (E3) and the synthetic estrogen 17α-ethinylestradiol (EE2) are among the most troublesome estrogens (AURIOL, FILALI-MEKNASSI, *et al.*, 2006, KOH, CHIU, *et al.*, 2008, KUSTER, LÓPEZ DE ALDA, *et al.*, 2008).

17β-estradiol is the main natural human estrogen and presents the greatest estrogenic potential, and this is the reason for being used as a positive control (standard) in the measurement of estrogenic activity in the Yeast Estrogen Screen (YES) assay (ROUTLEDGE, SHEAHAN, *et al.*, 1998). This hormone has important effects not only on the development and regulation of the female menstrual cycle, but also on the synthesis of bone tissue. While 17β-estradiol levels in men are lower compared to women, this hormone also plays an important role in male reproduction by preventing sperm cell apoptosis (PENTIKÄINEN, ERKKILÄ, *et al.*, 2000).

17α-ethinylestradiol is the main synthetic estrogen derived from 17β-estradiol. It was first synthesized in 1938 in Germany to be used as an oral contraceptive and to treat symptoms of menopause and female hypogonadism (TERNES, 1998). In synthetic estrogens, the addition of ethinyl and methyl groups to aromatic structures results in a more resistant molecule, with a high estrogenic potential (SERVOS, BENNIE, *et al.*, 2005, COMBALBERT, HERNANDEZ-RAQUET, 2010). Table 2.2 shows the chemical structures of the main natural estrogen E2 and the main synthetic estrogen EE2.

Table 2.2 – Chemical structures of the natural estrogen 17β-estradiol and the synthetic estrogen 17α-ethinylestradiol.

Compound	**Chemical structure**
17β-estradiol (E2)	
17α-ethinylestradiol (EE2)	

2.5.1. Physicochemical Properties

The knowledge of the main physicochemical properties of EDCs is important to understand their behavior in the environment and to predict the fate of these compounds after their release in water bodies.

Estrogens are non-volatile organic compounds with vapor pressures between $6x10^{-9}$ and $3x10^{-8}$ Pa. These compounds are poorly soluble in water, with solubility of 13 mg L^{-1} for E2 and 4.8 mg L^{-1} for EE2 (COMBALBERT, HERNANDEZ-RAQUET, 2010). The octanol-water partition coefficient (K_{OW}), defined as the ratio between the concentration of a substance in the octanol phase and its concentration in the aqueous phase in a two-phase octanol-water mixture, is an indicator of the hydrophobicity of a compound and can also determine its affinity by organic matter (CLOUZOT, MARROT, *et al.*, 2008). Log K_{OW} values between 3 and 5 are characteristic of hydrophobic substances, as in the case of estrogens E2 and EE2.

Additionally, the adsorption coefficient (K_{OC}) is another important parameter, as it defines the adsorption of a compound on organic carbon. The K_{OC} coefficient shows a relationship with the K_{OW} coefficient, where the greater the hydrophobicity of a compound the greater its affinity for organic matter (CLOUZOT, MARROT, *et al.*, 2008). Table 2.3 presents the main physicochemical properties of the estrogens used in this study.

Table 2.3 – Main physicochemical properties of estrogens E2 and EE2.

Compound	**Chemical formula**	**Molar mass (g mol^{-1})**	**Vapor pressure (Pa)**	**Water solubility at 20 °C (mg L^{-1})**	**Log K_{OW}**	**Log K_{OC}**
17β-estradiol (E2)	$C_{18}H_{24}O_2$	272.3	$3x10^{-8}$	13	3.94	3.51
17α-ethinylestradiol (EE2)	$C_{20}H_{24}O_2$	296.4	$6x10^{-9}$	4.8	4.15	3.68

2.5.2. Occurrence and Fate

Natural and synthetic estrogens are the most relevant EDCs found in the environment. In humans and other mammals, estrogens are metabolized first in the liver

and are later excreted, in large part, as inactive polar conjugates through urine and feces (RACZ, GOEL, 2009). Typical values of E2 excreted daily by women vary between 3 - 19 µg, and these values are increased to approximately 400 µg d^{-1} for women in pregnancy (COMBALBERT, HERNANDEZ-RAQUET, 2010). In men, the levels of E2 excreted are much lower, around 1.5-7 µg d^{-1} according to D'Ascenzo et al. (2003). On the other hand, the synthetic estrogen EE2 used as a contraceptive presents between 20 - 60 µg per tablet, which is ingested daily and approximately 50% of it is excreted naturally without being metabolized by the body (JOHNSON, WILLIAMS, 2004).

WWTPs are not designed to remove estrogens and other micropollutants. Consequently, they are not completely degraded during the biological processes, being released into the receiving bodies along with the treated effluent. Therefore, treated sewages are one of the main sources of EDCs in the aquatic environment (RACZ; GOEL, 2009; COMBALBERT; HERNANDEZ-RAQUET, 2010).

The detected concentrations of EDCs in the water are normally in the order of ng L^{-1}, with values ranging from 5 to 30 ng L^{-1} for E2 and below 10 ng L^{-1} for EE2 (TERNES, KRECKEL, *et al.*, 1999). Although these concentrations are low, studies have reported that even at these levels, estrogens can cause negative effects on the endocrine system of aquatic organisms, as fishes (BILA, DEZOTTI, 2007, CHANG, CHOO, *et al.*, 2009, YU, DEEB, *et al.*, 2013).

Due to its high affinity for organic matter and its hydrophobicity, a portion of the estrogens can be adsorbed onto biological sludge during the biological treatment, which can be digested for biogas generation, used as a fertilizer in agricultural activities or disposed in landfills. Nonetheless, estrogens present in the sludge, even after stabilization, can percolate into groundwater, aggravating the EDCs problem contamination (LUO, GUO, *et al.*, 2014).

Another recurring problem is associated to the release of treated domestic wastewaters into the receiving bodies containing low levels of EDCs. The water from rivers, bays and other aquatic resources is captured, treated and distributed for human consumption. However, water treatment plants are unable to completely remove these compounds resulting in drinking water with the presence of estrogenic substances (SCHÄFER; WAITE, 2002; AURIOL et al., 2006).

Although the concern of the international community has increased in the sense of creating laws that reduce the emissions of micropollutants into aquatic environments, in Brazil there is still no regulation that prohibits the discharge of these contaminants in

water. CONAMA Resolution 467 of 2015 specifies maximum values allowed for some metals, oils, and organic solvents, but not for other substances, allowing their release without any control.

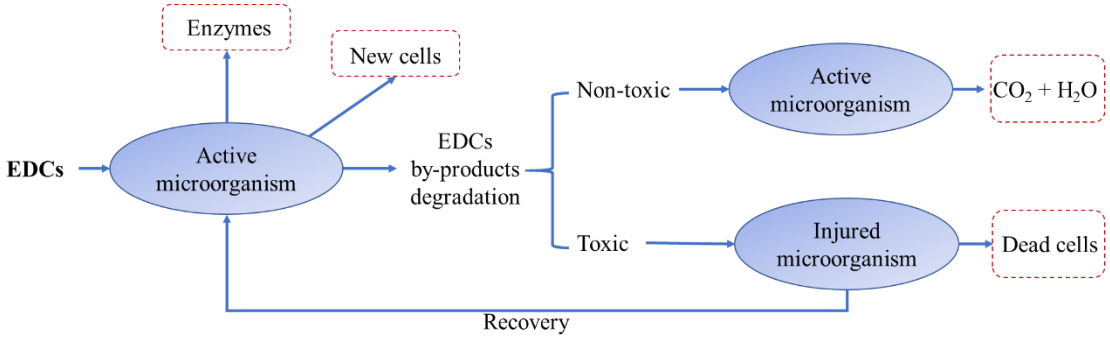
2.5.3. Methods for the Removal of EDCs

Different methods have been investigated to remove micropollutants, specifically EDCs present in water and wastewater. The main ones occur during secondary and tertiary treatment. Secondary or biological treatment has shown high efficiencies for EDCs removal due to the high affinity of estrogenic compounds for organic matter. Their hydrophobicity and the different microorganisms present in biological sludge can contribute favorably to the removal of these compounds.

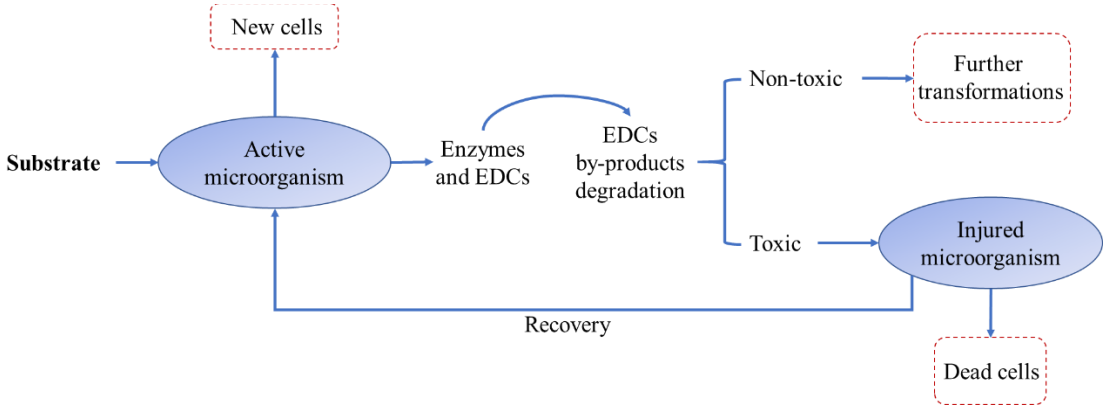
Different authors have studied the removal of estrogens by adsorption in activated sludge. Johnson and Sumpter (2001) reported that there was no complete removal of EDCs in activated sludge systems, while Andersen et al. (2005) found that the EDC removal by adsorption onto the sludge did not reach even 10%. Other authors argue that low removals are related to the characteristics of the wastewater, the sludge and reactor operational parameters such as HRT and SRT (CLARA, STRENN, *et al.*, 2004, PETRIE, MCADAM, HASSARD, *et al.*, 2014).

Another important factor that has been studied as one of the most important in the removal of EDCs using biological systems is the composition of the microbial community. In the research carried out by Khunjar et al. (2011) it was observed that ammonia-oxidizing bacteria have a five times greater contribution to the biodegradation of EE2 when compared to heterotrophic bacteria. The EDC biodegradation process has been the focus of recent studies, from which two possible main routes of degradation by the microorganisms were proposed: metabolism and co-metabolism (KHUNJAR, MACKINTOSH, *et al.*, 2011, TRAN, URASE, *et al.*, 2013, SONG, H. L., YANG, *et al.*, 2017).

In microbial metabolism, organic compounds (including EDCs) are used as a substrate and source of energy to perform cell growth and maintenance activities, while in co-metabolism, other compounds are used as an energy source for the enzymes production that may assimilate EDCs. Figure 2.9 depicts the two possible pathways of EDCs biodegradation.



(a)



(b)

Figure 2.9 – EDCs biodegradation pathways scheme: (a) microbial metabolism and (b) microbial co-metabolism. Adapted from Tran, Urase *et al.* (2013).

Estrogen metabolism is mainly carried out by heterotrophic bacteria, while co-metabolism is performed by ammonia-oxidizing bacteria, mainly *Nitrosomonas*, *Nitrospira* and *Nitrobacter*, which use the ammonia monooxygenase (AMO) enzyme to degrade EDCs (SKOTNICKA-PITAK, KHUNJAR, *et al.*, 2009, KHUNJAR, MACKINTOSH, *et al.*, 2011). Khunjar et al. (2011) suggest that higher rates of EDCs removal can be achieved when nitrifying bacteria act in conjunction with heterotrophic bacteria.

However, few studies have reported EDCs removal in AGS-based systems. Balest et al. (2008) compared the removal efficiencies of E1 and E2 using activated sludge and AGS and reported that greater estrogen removals were achieved in the latter, around 60% for E1 and 69% for E2. Zheng et al. (2015) compared the adsorption capacity of E2 in granular sludge and in activated sludge. These authors reported that the aerobic granules presented better conditions for the adsorption of E2. On the other hand, Margot et al. (2016) used AGS to evaluate the removal of 36 micropollutants, including: E1, E3 and

bisphenol. Two SBR were used, with and without nitrification, and most of the contaminants were quickly biodegraded (approximately 80%) during the aerated phase than during the anoxic/anaerobic phase. This fact suggests that most of the EDCs were degraded by heterotrophic microorganisms. Nitrifying microorganisms also contributed to the biodegradation, but in lower proportion, proving the theory proposed by Khunjar et al. (2011).

Tertiary treatment processes have been evaluated for the removal of EDCs. Adsorption on activated carbon has shown high removals from synthetic wastewaters (WESTERHOFF, YOON, *et al.*, 2005, SNYDER, ADHAM, *et al.*, 2007). However, in the treatment of real wastewaters, there is a competition between EDCs and other compounds for the active sites of the adsorbent, consequently a reduction in removal efficiencies (LUO, GUO, *et al.*, 2014). The main disadvantage of the adsorption process is the need for continuous recovery of the adsorbent (e.g., activated carbon), reflecting in an increase in the operating costs and energy consumption (KOH, CHIU, *et al.*, 2008). Membrane separation processes have also been studied to remove estrogenic substances, but they have shown low efficiencies (around 40%) (SNYDER, ADHAM, *et al.*, 2007), which can be improved by coupling them with biological treatment in membrane bioreactors (CHANG, CHOO, *et al.*, 2009, OJAJUNI, SAROJ, *et al.*, 2015).

Several studies have addressed the removal of EDCs using different Advanced Oxidation Processes (AOPs), which have shown excellent removal efficiencies. Among the most common AOPs are: ozonation (ESPLUGAS, BILA, *et al.*, 2007, MANIERO, BILA, *et al.*, 2008, LARCHER, DELBÈS, *et al.*, 2012, REUNGOAT, ESCHER, *et al.*, 2012), photocatalysis (ZUO, ZHANG, *et al.*, 2006, BELGIORNO, RIZZO, *et al.*, 2007, BENOTTI, STANFORD, *et al.*, 2009, SARASIDIS, PLAKAS, *et al.*, 2014, CASTELLANOS, BASSIN, *et al.*, 2020), Fenton process (IFELEBUEGU, EZENWA, 2011, DE LA CRUZ, GIMÉNEZ, *et al.*, 2012), electrochemical oxidation (MOREIRA, SOLER, *et al.*, 2016, HUA, HE, *et al.*, 2019) and oxidation by persulfate (ZHANG, B. T., ZHANG, *et al.*, 2015, WANG, J., WANG, 2018, ZHOU, Z., LIU, *et al.*, 2019). Nevertheless, the main concern in the use of POAs in the removal of EDCs is the partial oxidation and the possibility of by-products formation with similar or even greater estrogenic potential than the original compounds.

2.6. Advanced Oxidation Processes (AOPs)

The advanced oxidation processes are extremely efficient technological alternatives used in the treatment of wastewaters, mainly for the removal of hardly biodegradable organic and inorganic substances which are often in low concentrations. The AOPs allow the mineralization of pollutants into CO_2, H_2O and inorganic ions or their transformation into less complex products by means of hydroxyl radicals (•OH), which are strong oxidizers with high oxidation potential, capable of destroying organic molecules present in several aqueous matrices (BILA, AZEVEDO, *et al.*, 2008). The hydroxyl radical is the most reactive oxidizing agent employed in wastewater treatment, with an oxidation potential between 1.95 - 2.8 eV and a nonselective behavior. Given its inherent characteristic, it quickly reacts with numerous species at rate constants in the order of 10^8 to 10^{10} M^{-1} s^{-1} (STASINAKIS, 2008). Due to their very short lifetime, •OH are only produced in situ through the application of different methods, including oxidizing agents, such as H_2O_2 and O_3, ultraviolet light irradiation, and catalysts such as Fe^{2+} and TiO_2 (DENG, ZHAO, 2015).

Fundamentally, the AOPs are classified in two main groups: homogeneous processes, for which the system is composed by one phase; and heterogeneous processes, with more than one phase and in which the reaction generally takes place in the interface. The classification of the AOPs is presented in Figure 2.10.

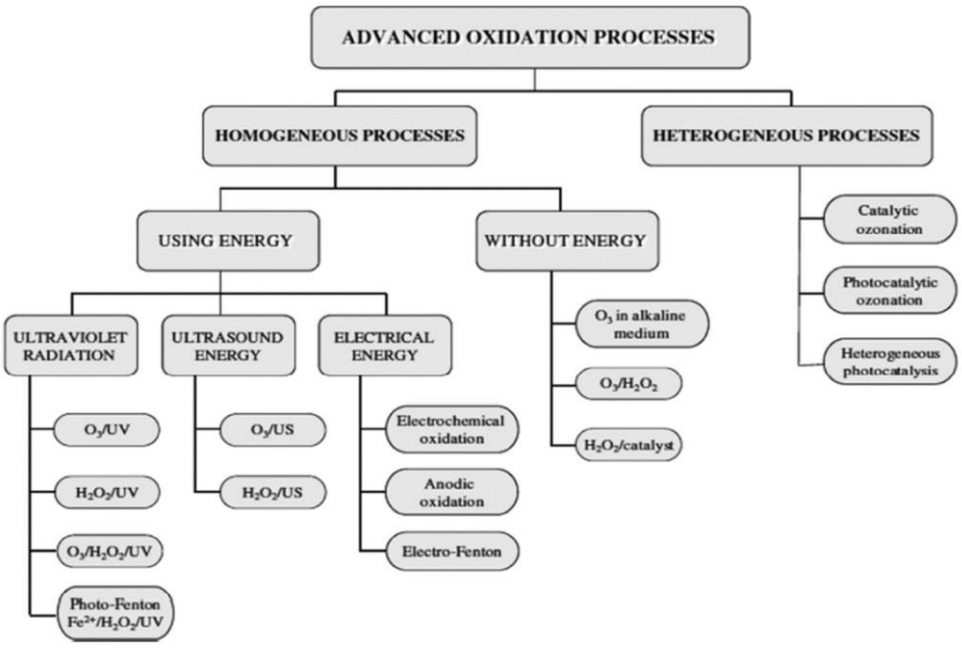


Figure 2.10 – Advanced oxidation processes classification (MOKHBI, KORICHI, *et al.*, 2019).

2.6.1. Heterogeneous Photocatalysis

The term photocatalysis consists in the combination of the photochemistry and catalysis processes which implies that light and a catalyst are necessary to lead a chemical reaction. Photocatalysis can be classified as homogeneous or heterogeneous according to the difference of phases between the catalyst and the reacting species (KUMAR, BANSAL, 2013). Heterogeneous photocatalysis is a chemical oxidation process in which a metal oxide semiconductor immersed in water is irradiated by ultraviolet (UV) light source resulting in an electron promoted, from the valence band to the conduction band, forming free hydroxyl radicals (•OH) (BELGIORNO, RIZZO, *et al.*, 2007).

The most common semiconductors or catalysts used are: TiO_2, Fe_2O_3, ZnO, WO_3, and BiO_3, being TiO_2 largely employed due to its properties: it is chemically and biologically inert, photocatalytically stable, relatively easy to produce and to use, able to efficiently catalyze reactions, exhibit low cost and low risks to environment or humans (VAN GERVEN, MUL, *et al.*, 2007).

The photocatalytic process starts by the absorption of a photon (hv) with an energy equal or higher than the semiconductor band-gap energy (E_{bg}), normally 3.2 eV. This energy supplied by UV light induces the transference of a negative electron (e_{CB}^-) from the catalyst valence band (VB) to the conduction band (CB), leaving a positive site termed "electron hole" (h_{VB}^+) in the valence band with oxidative properties. The photogenerated electrons (e_{CB}^-) are immediately trapped by dissolved molecular oxygen present in the water forming superoxide radical anions ($O_2^{\bullet -}$), while the holes can be trapped by hydroxide ions or water adsorbed at the semiconductor surface to generate hydroxyl radicals (SANTOS, PAULISTA, *et al.*, 2019). The general reactions for the heterogeneous photocatalysis process are shown in Reactions (2.10) - (2.12).

$$semiconductor + hv \rightarrow e_{CB}^- + h_{VB}^+ \quad (2.10)$$

$$e_{CB}^- + O_{2_{ads}} \leftrightarrow O_2^{\bullet -} \quad (2.11)$$

$$h_{VB}^+ + OH^- \leftrightarrow HO^\bullet \quad (2.12)$$

2.6.2. UV/H_2O_2 Process

Hydrogen peroxide (H_2O_2) is a chemical compound commonly used as a bleaching agent, antiseptic and oxidant. At the WWTPs, H_2O_2 has been used as a disinfectant and also to remove low levels of pollutants from wastewaters as chlorine, nitrites, sulphites and hypochlorites (KUMAR, BANSAL, 2013).

When combining H_2O_2 with UV light, its oxidizing power can be considerably improved, due to the cleavage of O-O bond, forming two moles of •OH radicals from the photolysis of one mole of H_2O_2 (VILAR, ALFONSO-MUNIOZGUREN, *et al.*, 2020), as shown in Equation (2.13).

$$H_2O_2 + hv \rightarrow 2HO^{\bullet} \quad (2.13)$$

Wavelengths lower than 280 nm are efficiently use to cleave the O-O bond in the H_2O_2 molecule, which is the reason to normally employ UVC radiation to promote the photolytic reaction. The rate of photolysis in aqueous H_2O_2 is dependent on the pH and increases when more alkaline conditions are used (KUMAR, BANSAL, 2013).

The use of H_2O_2 as an oxidant offers a wide number of advantages when compared with other methods for chemical or photochemical wastewater treatment, such as: infinite solubility in water, ease of being found commercially, thermal stability, relatively low cost and easy storage. On the other hand, there are some disadvantages associated with H_2O_2 use, mainly related to its small molar absorption coefficient and the need for removing the excess concentration from water because it can be toxic at high levels (PABLOS, MARUGÁN, *et al.*, 2013).

2.6.3. Combined UV/H_2O_2/TiO_2 Process

The combination of two or more AOPs have proved to be appropriate for the removal of contaminants present in the water due to the synergic effect, improving the individual performance of the individual processes (VILAR, ALFONSO-MUNIOZGUREN, *et al.*, 2020). Thus, the coupling between UV/H_2O_2 and photocatalysis brings a greater efficiency in the oxidation processes due to the increase in the production of hydroxyl radicals (ESPÍNDOLA, SZYMAŃSKI, *et al.*, 2019). Figure 2.11 presents a diagram with the different mechanisms for the •OH radicals formation when UV/H_2O_2 is combined with heterogeneous photocatalysis using TiO_2 as a catalyst.

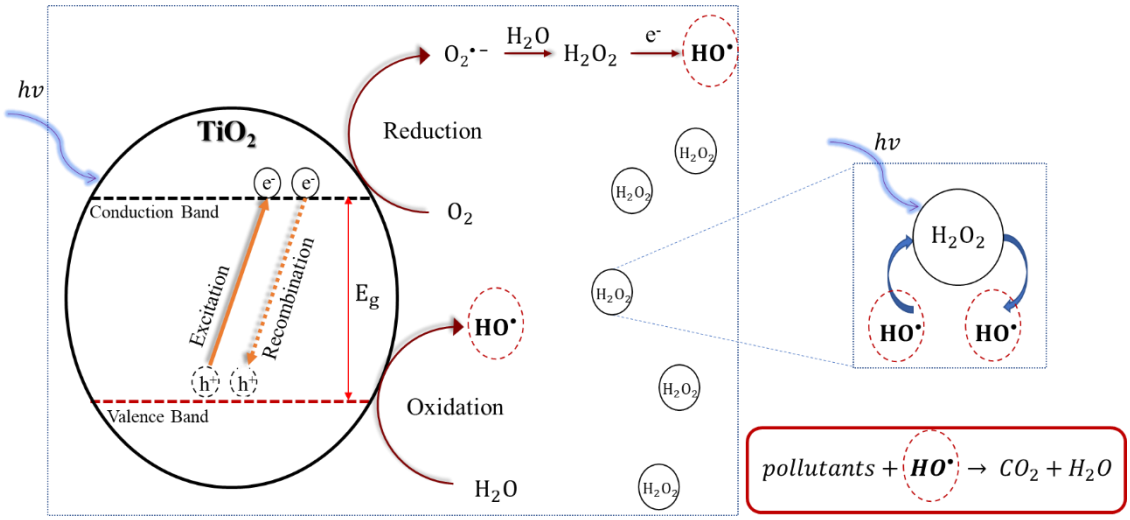


Figure 2.11 – Hydroxyl radical generation mechanisms in the combination of photocatalysis and UV/H_2O_2 processes. Source: author.

Despite the advantages of the combined processes, there are still some problems that need to be solved for the implementation of the photocatalytic oxidation reactors for wastewater treatment at large scales. The modeling, simulation, design, scale-up and optimization of photocatalytic reactors is difficult because it simultaneously involves hydrodynamics, mass transfer, chemical reaction and irradiance along with other conventional reactor complications such as pollutant catalyst contact time, temperature control, and catalyst installation (KUMAR, BANSAL, 2013). Some issues related to the UV radiation source including low life time, power instability, low photonic efficiency also must be considered. An alternative to the conventional UV sources is the use of solar light. However, this implies larger installation spaces and the efficiencies are associated to factors such as intensity, direction and availability of the solar light (GRILLA, MATTHAIOU, *et al.*, 2019).

The present thesis consists of three works, being the first intended to evaluate the effect of the solids retention time on the removal of organic matter and nutrients (N and P) in a AGS SBR. The second work addressed the simultaneous removal of EDCs and nutrients in the same reactor technology, along with the evaluation of estrogenic activity. Finally, the third work refers to the study of the removal of EDCs through heterogeneous photocatalysis with the addition of H_2O_2 in a membrane micro-reactor.

3. Effect of Sludge Age on Aerobic Granular Sludge: Addressing Nutrient Removal Performance and Biomass Stability

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3.1. Introduction

Aerobic granular sludge (AGS) technology has demonstrated numerous advantages over conventional activated sludge-based wastewater treatment systems. AGS is based on the formation of self-aggregated microbial agglomerates which show excellent settling properties (WANG, X. H., ZHANG, *et al.*, 2007, ADAV, LEE, 2008). Such characteristic makes solid-liquid separation easier, and, depending on the reactor configuration, there is no need for secondary clarifiers, leading to a significant reduction in plant footprint and operational costs (PRONK, DE KREUK, *et al.*, 2015, BASSIN, TAVARES, *et al.*, 2019). Besides, due to the coexistence of different redox layers (aerobic and anoxic/anaerobic) within the granules, biological nitrogen and phosphate removal can be simultaneously achieved (MOY, TAY, *et al.*, 2002, DE KREUK, HEIJNEN, *et al.*, 2005, BASSIN, KLEEREBEZEM, *et al.*, 2012b).

Conventional biological nitrogen removal is generally obtained by aerobic nitrification and anoxic denitrification, whereas the removal of phosphate can be accomplished through the enhanced biological phosphate removal (EBPR) under alternating anaerobic-aerobic (anoxic) conditions (MOSQUERA-CORRAL, DE KREUK, *et al.*, 2005, BASSIN, KLEEREBEZEM, *et al.*, 2012a). Polyphosphate-accumulating organisms (PAOs) are regarded as the responsible for the EBPR process. To favor the development and growth of these organisms and therefore obtain stable phosphorus removal over time, some operational strategies may be implemented. One of them include the application of a feast-famine regime in the reactor (WAN, J., BESSIÈRE, *et al.*, 2009), which also enhances granular stability (DE KREUK, VAN LOOSDRECHT, 2004). This strategy consists in the incorporation of an anaerobic feeding phase in the SBR cycle, during which organic carbon is taken up by PAOs and stored as intracellular polymers (namely polyhydroxyalkanoates - PHA) (LOPEZ-VAZQUEZ, OEHMEN, *et al.*, 2009). Concomitantly, phosphate is released from the cells to the bulk liquid. Subsequently, an aerated phase takes place (DE KREUK, VAN LOOSDRECHT, 2004). Under aerobic or anoxic conditions, PAOs use the energy provided by the oxidation of PHA to accumulate phosphorus as intracellular polyphosphate and grow (FLOWERS, HE, *et al.*, 2009). For this purpose, oxygen is used in the outer layer of the granules while nitrate or nitrite in their inner zone (DE KREUK, PRONK, *et al.*, 2005). In the latter case, phosphate removal and denitrification are combined, in the so-called denitrifying dephosphatation process (BASSIN,

KLEEREBEZEM, *et al.*, 2012a). Nevertheless, the use of a feast-famine regime may also stimulate the growth of glycogen-accumulating organisms (GAOs), which compete with PAOs for the available organic source but do not contribute for the phosphorus removal process (WINKLER, BASSIN, *et al.*, 2011). To avoid deterioration of phosphate removal in EBPR systems, strategies to limit the growth of GAOs have been addressed in previous investigations (LOPEZ-VAZQUEZ, HOOIJMANS, *et al.*, 2009, WEISSBRODT, MAILLARD, *et al.*, 2014). At the oxygen-containing outer part of the granules, nitrification also occurs generating the oxidized nitrogen compounds (NO_x) which are reduced to N_2 in the anoxic regions via denitrification.

Nitrogen and phosphate removal processes are strongly dependent on the sludge retention time (SRT). If sludge discharge is not regularly performed, the growth of PAOs can be compromised (MULKERRINS, DOBSON, *et al.*, 2004, LI, N., WANG, *et al.*, 2008). The periodic removal of excess sludge from the reactor stimulates biomass turnover and the growth of new cells, and thus the metabolic and consumption rates of both organics and nutrients occurs faster (WINKLER, BASSIN, *et al.*, 2011). Some studies have shown that the biological removal of phosphorus is favored at SRT varying between 20 and 30 days (LEE, D., KIM, *et al.*, 2007, LI, D., LV, *et al.*, 2016), while others claim that low SRTs are more beneficial for this process (WINKLER, KLEEREBEZEM, *et al.*, 2012). Therefore, there no clear consensus regarding the optimum value of SRT to be applied in AGS reactors. On the other hand, a minimum sludge age is required for nitrification, given that nitrifying bacteria exhibits relatively low growth rates (CYDZIK, WOJNOWSKA, 2011). In this scenario, a compromise solution must be found in order to keep the desired microbial consortia inside the reactor, satisfying the requirements for both nitrogen and phosphate removal processes.

Besides affecting nutrient removal capability, the sludge age is a key factor influencing cell growth, biomass turnover and physical properties of AGS. One of the main problems encountered during the operation of AGS systems is the proliferation of filamentous organisms. Filamentous outgrowth leads to a decrease in granule density (bulking effect) and dramatically impairs its settling properties (MORGENROTH, SHERDEN, *et al.*, 1997, MOURA, DUARTE, *et al.*, 2018). Consequently, substantial biomass loss may occur, leading to process failure. The development of filamentous organisms is intrinsically related to the type and concentration of substrate consumed by the microorganisms (TAY, LIU, *et al.*, 2001, FIGUEROA, VAL DEL RIO, *et al.*, 2015), but the applied sludge age exerts a great influence on this process. As reported in previous

studies, high SRT may favor filamentous outgrowth in aerobic granules (LIU, Y., LIU, 2006, FIGUEROA, VAL DEL RIO, *et al.*, 2015). Therefore, removal of excess sludge and proper control of this parameter becomes essential to avoid these filamentous bulking organisms in AGS-based systems (BASSIN, KLEEREBEZEM, *et al.*, 2012b). Despite the importance of this sludge age to achieve both stable granulation and effective nutrient removal in AGS systems, there is no consensus about the most appropriate sludge age that enables high nitrogen and phosphate removal to be achieved while keeping granular structure stable over time, especially under tropical climate conditions. Most studies on aerobic granular were carried out at moderate to low temperatures (DE KREUK, PRONK, *et al.*, 2005, BAO, YU, *et al.*, 2009, PRONK, ABBAS, *et al.*, 2015, JIANG, Y., SHANG, *et al.*, 2016), making further investigations at higher temperatures important for spreading AGS the technology worldwide.

Therefore, the aim of this study was to investigate the effect of the sludge age on organic matter and nutrient removal performance by AGS and granule stability at temperatures ranging from 20 to 25 ºC. The relationship between the applied SRT and important granular biomass properties such as sludge volume index, sedimentation velocity, size distribution and sludge yield coefficient has also been addressed. To fulfil the study objectives, experiments were carried out on a long-term basis (over 392 days) while additional batch tests under were conducted periodically under different redox conditions for better comprehension of the conversions taking place within the granules. The granular sludge structure was regularly examined by microscopic analysis, whereas the identification and quantification of specific microbial functional groups playing a key role on the treatment process was assessed by fluorescence in situ hybridization analysis.

3.2. Materials and Methods

3.2.1. Reactor set-up and operating conditions

Experiments were performed in a lab-scale column-type aerobic granular sludge (AGS) sequencing batch reactor (SBR). Granules collected from another lab-scale reactor running on synthetic wastewater and subjected to SRT of 30 days were used as inoculum. At that time, this reactor was characterized by low phosphorus removal and the granules exhibited a significant amount of filamentous bacteria. The system used in this research

had a working volume of 1.5 L, an internal diameter of 5 cm and useful height of 79 cm. Reactor was operated in 3 h-cycle under alternating anaerobic and aerobic conditions in order to promote nitrogen and phosphate removal. The cycling profile comprised an anaerobic feeding phase of 60 min from the bottom of the reactor in a plug-flow regime through the settled sludge bed, 112 min aeration, 3 min settling and 5 min effluent withdrawal. Effluent was discharged 29 cm above the reactor bottom at a volume exchange ratio of 63%, resulting in a hydraulic retention time of 4.7 h. A Programmable Logic Controller (PLC) coupled with Versapro data acquisition software was used to control and operate the SBR.

The reactor was operated at room temperature (20 ± 3 ºC). Aeration and mixing were supplied through an air diffuser placed at the bottom of the reactor (airflow rate of 2.2 L min^{-1}). Average dissolved oxygen (DO) concentration within the aeration phase was kept at 5 mg L^{-1}, but it varied along the SBR cycle as follows: during the anaerobic feeding, no oxygen was present; at the end of aeration, DO reached a maximum of 6.6 mg L^{-1}; and during the settling phase (aeration off) it corresponded to 0.1 mg L^{-1}.

The AGS bioreactor was fed with a synthetic medium to allow more controlled conditions, maintaining the characteristics of the influent as desired. It consisted of two solutions with the following composition (concentrations in mM): (A) $NaCH_3COO.3H_2O$ 31.5 mM, $MgSO_4.7H_2O$ 1.8 mM, KCl 2.3 mM, $CaCl_2$ 3.3 mM and (B) NH_4Cl 21.4 mM, K_2HPO_4 2.1 mM, KH_2PO_4 1.1 mM and 5 mL L^{-1} trace element solution (VISHNIAC, SANTER, 1957). A volume of 158.3 mL from both solutions were combined with 633.2 mL of tap water in order to achieve the following influent concentrations: 400 mg L^{-1} of COD, 50 mg NH_4^+-N L^{-1}, 16 mg PO_4^{3-}-P L^{-1}. A mixed sample of excess biomass was removed from the sludge bed during the settling phase to obtain the desired sludge retention time (SRT). The reactor operation was divided in four different experiments runs (Table 3.1), during which the SRT was changed to evaluate its effect on reactor performance and granular sludge stability. This parameter was determined according to the method proposed in a previous study (WINKLER, BASSIN, *et al.*, 2011).

During run I, which lasted 49 days, the SRT was not intentionally controlled, being dependent on the natural biomass washout taking place during the effluent withdrawal phase. Afterwards, the SRT was maintained at around 30 days (run II) by periodically removing sludge from the reactor every two days. The SRT was then decreased to 20 days (run III) and finally to 15 days (run IV).

Table 3.1 – Experimental conditions of the aerobic granular sludge (AGS) sequencing batch reactor.

Experimental conditions	Sludge age (days)	Operating days in each run	Total duration of each run (days)[b]
I	Not controlled[a]	0 - 49	49
II	30	50 - 137	87
III	20	138 - 263	125
IV	15	264 - 392	128

[a]The sludge age (i.e., SRT) varied according to the amount biomass naturally leaving the reactor during the withdrawal phase (after the short settling period of 3 min). Taking into account the solids results, the sludge age was observed to vary between 47 and 61 days.
[b]Different duration of the experimental runs can be explained by the time needed to achieve pseudo-stationary conditions in each one (i.e., stable COD, N and P conversions).

3.2.2. Cycle tests

Typical cycle tests were carried out when a pseudo-steady-state condition was achieved at the different operational runs. Liquid samples were collected every 10 to 20 min only during aeration phase, when the reactor content was mixed. The first sample was taken 2 min after aeration period has started (i.e., 62 min of the operating cycle) to allow enough mixture. Ammonium and phosphate uptake rates were determined by linear regression of their concentrations over time divided by the volatile suspended solids (VSS) concentration in the reactor. The denitrification rate was estimated based on the difference between the ammonium uptake rate and the nitrite and nitrate production rate observed during nitrification in a typical cycle, as suggested by Bassin et al. (2012c).

3.2.3. Additional experiments

During normal operation of the AGS reactor, aerobic and anoxic P-uptake simultaneously occur in different zones of the granules, so that it is impossible to distinguish the phosphate taken up with oxygen or nitrite/nitrate as electron acceptor. Therefore, in order to estimate the maximum anoxic phosphate uptake capacity, evaluate its contribution for the overall phosphate removal and obtain information on the key players in the denitrification process, additional cycle tests were also carried out under anoxic conditions, achieved by bubbling only nitrogen gas instead of air in the reactor.

These tests were performed during run III, in which EBPR performance has been improved in the AGS reactor. In the anoxic cycle tests, electron acceptor (nitrate) was continuously dosed according to the denitrification rate estimated from the SBR cycle tests (as described in section 3.2.2). Additional tests dosing electron acceptor at twice as the denitrification rate were also performed.

Therefore, batch experiments and cycle tests were performed under fully anoxic conditions in order to determine the maximum anoxic P-uptake capacity. This is important for instance to estimate the potential significance of anoxic removal of phosphate linked to nitrogen removal by denitrifying PAOs (i.e., DPAOs) via denitrifying dephosphatation.

3.2.4. Determination of physical properties of the granules

Average diameter of the granules from the sludge bed was determined by using *Image J* analyzer software. Biomass density was measured with a pycnometer (WINKLER, BASSIN, *et al.*, 2011). The sludge volume index (SVI) is a parameter that indicates the volume occupied per mass unit (mL $gTSS^{-1}$) of sludge after settling for 5 (SVI_5) and 30 min (SVI_{30}), and was determined according to Schwarzenbeck; Erley; Wilderer, (2004) and Standard Methods (APHA - AMERICAN PUBLIC HEALTH ASSOCIATION, 2005). The settling velocity of the granules was determined experimentally, taking into account the time that a representative biomass sample (composed of different-sized granules) took from the top to the base a cylinder of 1L. Then, Equation (3.1) was used to determine the average settling velocity of the granular particles.

$$V_m = \frac{d}{t_m} \tag{3.1}$$

Where V_m is the average settling velocity (m s^{-1}), d is the distance traveled by the particles (m) and t_m is the average time spent to travel the defined distance (s).

3.2.5. Analytical measurements

COD, ammonium-nitrogen (NH_4^+-N), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N) and phosphate (PO_4^{3-}-P) concentrations were determined according to Standard Methods (APHA - AMERICAN PUBLIC HEALTH ASSOCIATION, 2005). The total suspended solids (TSS) and volatile suspended solids (VSS) inside the reactor were quantified using the methodology described by Bassin et al. (2012a). The method proposed is more appropriate for AGS systems since the granular biomass tend to be more concentrated in the lower parts of the reactor, so that a completely mixed sludge sample cannot be obtained, as is the case of flocculent sludge reactors. In this method, samples of 5 mL of granules were collected during the aerated phase in the reactor and put into a volumetric cylinder. The samples were dried at 105 °C for 24 h until constant weight was obtained (TSS content). Afterwards, samples were placed in a muffle at 560 °C for 1 h to determine the ash content (fixed suspended solids – FSS). VSS content was then obtained by subtracting the FSS from the TSS. The TSS and VSS in the effluent were quantified using conventional thermogravimetric methods (APHA - AMERICAN PUBLIC HEALTH ASSOCIATION, 2005). Samples of 100 mL of effluent were collected during the withdrawal phase and filtered over a 0.45 µm glass filter. Subsequently, the same procedure employed for determination of TSS and VSS concentrations inside the reactor was used.

3.2.6. Fluorescence in situ hybridization (FISH)

For FISH analysis, samples of granules were removed from the reactor during the aerated phase (to ensure homogeneity). First, the biomass fixation was carried out as follows: the liquid present in the sample was removed manually leaving only the granules, which were gently macerated and subsequently centrifuged at low speed for 1 min. The supernatant was removed and the biomass was transferred to a 2 mL Eppendorf tube which was again centrifuged at 1400 RPM for 2 min. The supernatant was removed and phosphate-saline buffer (PBS) with pH = 7.2 was added. The sample was stirred for resuspension and centrifuged, and this step was repeated for 3 times. Subsequently, paraformaldehyde 4% (v/v) was added and the sample was refrigerated for 2 hours. After this time, the sample was washed again with PBS buffer 3 times and finally resuspended

in a PBS/ethanol (98%) solution (1:1) to be stored at -20 °C until it was analyzed. To prepare the slides for hybridization, 100 mL of distilled water, 0.1 g of microbiological gel and 0.01 g of $KCr(SO_4)_2$ were used with drying at a temperature of 48 ºC. The sample, previously fixed, was spread in the slide and placed in an oven for drying during 15 minutes. Then the cells contained in each slide well were dehydrated gradually in a three-stage procedure (lasting 5 min each), with increasing concentrations of ethanol (i.e., 50%, 70% and 98% (v/v)). After dehydration, 10 μL of hybridization buffer (NaCl 5 M, Na_2EDTA 0.5 M, Tris/HCl 1 M, sodium dodecyl sulfate (SDS) 10% (v/v), and formamide 30% (v/v)), and 1 μL of each oligonucleotide probe marked with different fluorochromes (green-fluorescent dye Alexa Fluor 488 and red-fluorescent dye Alexa Fluor 594) at final concentration of 5 ng μL^{-1} were added to each slide well. Oligonucleotide probes, their respective sequences and target groups are shown in Table 3.2. Some probes were combined to identify a target functional group (e.g., PAOs, GAOs, ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB)).

Hybridization was carried out for 16 hours at a temperature of 46 ºC in a dark incubation chamber containing a hybridization buffer-saturated tissue paper below the slide. After hybridization step, the slide was washed with a washing buffer (NaCl 5 M, Na_2EDTA 0.5 M, Tris/HCl 1 M, sodium dodecyl sulfate (SDS) 10% (v/v)) solution at 48ºC for 20 min, in order to remove the excess (non-hybridized) probe. Finally, the slide was washed with distilled water and introduced again in the oven for drying. This step was followed by addition of Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) to all slide wells to preserve fluorescence and stored at -20 ºC. The slides were then observed in a LSM 710 laser scanning confocal microscope, from Carl Zeiss.

Granular biomass samples taken from the reactor operated under a certain operational condition was analyzed in triplicate, in different wells of the FISH slides. Six images were obtained from each well, totaling 18 images for each sample. The images were obtained using the Zeiss Black Software, in czi format, and exported to the *Python Jupyter* program in order to avoid loss of information. The quantitative analysis was done through specific probes for the target group, in relation to the total bacterial community.

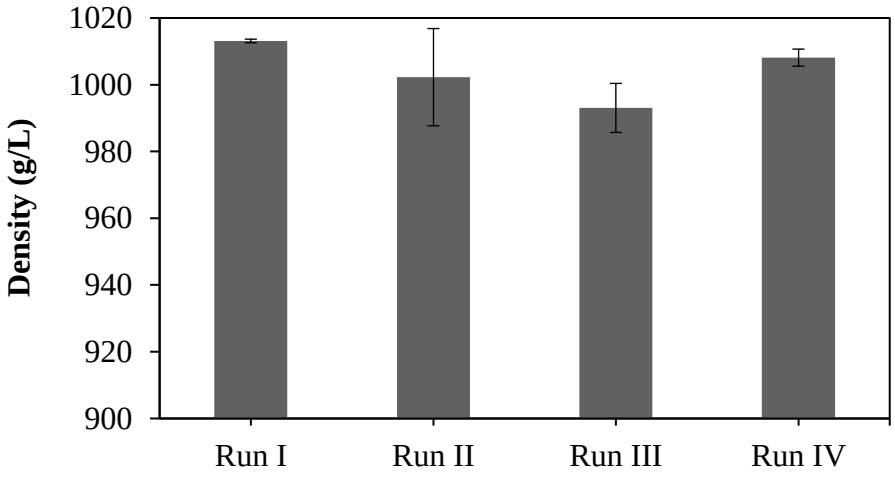
Table 3.2 – Oligonucleotide probes, their respective sequences and target groups.

Probe	Sequence (5'-3')	Target group	Probe combination (mix)	Reference
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobia*		Daims et al. (1999)
PAO 462	CCGTCATCTACWCAGGGTATTAAC	*Accumulibacter*	PAOmix	Crocetti et al. (2000)
PAO 651	CCCTCTGCCAAACTCCAG	*Accumulibacter*		Crocetti et al. (2000)
PAO 846	GTTAGCTACGGACTAAAAGG	*Accumulibacter*		Crocetti et al. (2000)
GAO Q431	TCCCCGCCTAAAGGGCTT	*Competibacter*	GAOmix	Crocetti et al. (2002)
GAO Q989	TTCCCCGGATGTCAAGGC	*Competibacter*		Crocetti et al. (2002)
Neu 653	CCCCTCTGCTGCACTCTA	*Nitrosomonas spp.*	AOBmix	Wagner et al. (1995)
Nse 1472	ACCCCAGTCATGACCCCC	*Nitrosomonas*		Mobarry et al. (1996)
Nso 1225	CGCCATTGTATTACGTGTGA	*β-proteobacteria*		Mobarry et al. (1996)
Nmv	TCCTCAGAGACTACGCGG	*Nitrosococcus m.*		Pommerening-Roser et al. (1996)
Nso 190	CGATCCCCTGCTTTTCTCC	*β*-proteobacteria		Mobarry et al. (1996)
Nit 1035	CCTGTGCTCCATGCTCCG	*Nitrobacter spp.*	NOBmix	Wagner et al. (1996)
Ntspa 662	GGAATTCCGCGCTCCTCT	*Nitrospira*		Daims et al. (2001)

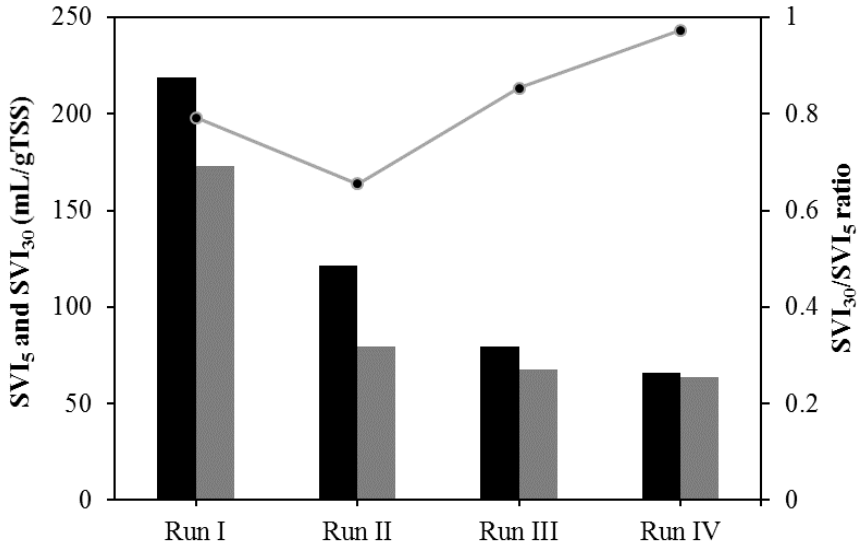
3.3. Results and Discussion

3.3.1. Aerobic granular sludge reactor (AGS) properties

The sludge used as inoculum for the AGS reactor start-up already contained a certain number of filaments, as described previously. At the beginning of run I, the average density of the granules was 1012 g L^{-1} (Figure 3.1a), a value expected for stable granules (WINKLER, KLEEREBEZEM, *et al.*, 2013), while the average settling velocity was 61 m h^{-1}. However, at the end of run I, the density started to decrease and the physical appearance of the granules changed as compared to that shown by the inoculum sludge, acquiring a darker colour and a non-compact structure. The average values for SVI_5 (sedimentation after 5 min) and SVI_{30} (sedimentation after 30 min) during the run I were 218 and 173 mL $gTSS^{-1}$, respectively (Figure 3.1b). These values are considered high for AGS, giving a ratio SVI_{30}/SVI_5 of 0.7, characteristic of poor settling and unstable granular biomass.



(a)



(b)

Figure 3.1 – a) Average density of the granules and b) sludge volumetric index (SVI) for 5 min (■), 30 min of sedimentation (■) and SVI_{30}/SVI_5 ratio (●) within each operational phase.

Furthermore, an abundant number of filaments around the granules was still observed (Figure 3.2).

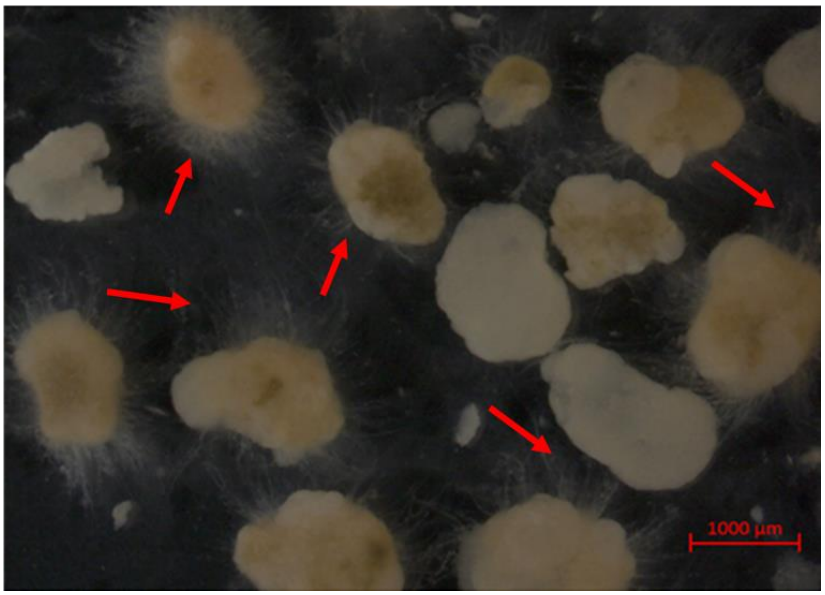
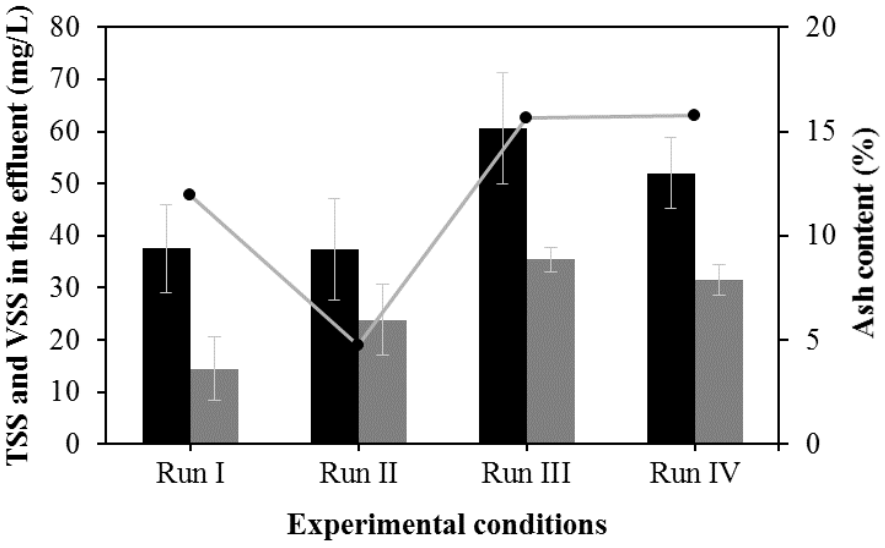


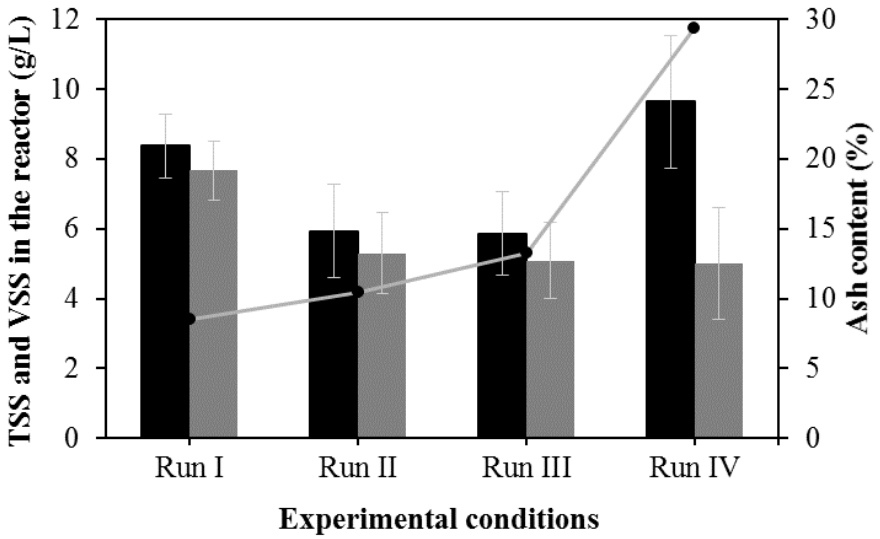
Figure 3.2 – Image of the granular sludge obtained in a stereoscopy after 50 days of operation. The red arrows indicate the presence of filaments around the granules.

This decrease in density is related to the presence of filaments in the granules, which directly affects their sedimentation properties. Within run II, during which the SRT started to be controlled at 30 days, the average density of the granules was lower than in run I (1002 g L^{-1}) and filaments were still apparent in the granules.

Over this stage, the SVI_{30}/SVI_5 radio decreased to 0.65 and, as a result of the density decrease, the settling velocity drastically dropped to 45 m h^{-1}. In run III (SRT of 20 days), the average density reached its minimum value (993 g L^{-1}) (Figure 3.1a). Concomitantly, an increment in total suspended solids (TSS) concentration in the effluent was observed, reaching 61 mg L^{-1} (Figure 3.3a).



(a)



(b)

Figure 3.3 – Average total suspended solids (TSS) (■), volatile suspended solids (VSS) (■) and ash content (●) a) in the effluent and b) within the reactor during each operational phase.

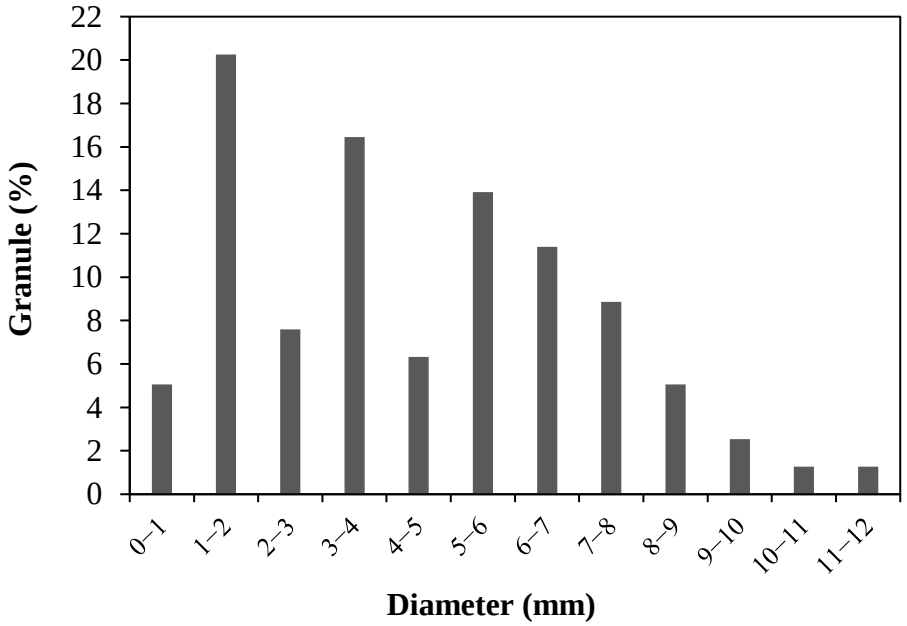
At the end of run III, the density of the granules started to increase and the biomass characteristics have changed, assuming a whiter appearance and a more defined structure. SVI_5 and SVI_{30} showed similar values (79 and 68 mL $gTSS^{-1}$), leading to an increase of SVI_{30}/SVI_5 ratio to 0.85, which indicated better biomass settling properties. With the sludge age being controlled and maintained at 15 days (run IV), the filaments of the granules began to disappear and therefore the average density increased to 1008 g L^{-1} (Figure 3.1a). Consequently, the SVI_5 and SVI_{30} average values were the lowest, i.e., 65 and 64 mL $gTSS^{-1}$, respectively, giving a SVI_{30}/SVI_5 ratio of 0.97 (Figure 3.1b), a value associated with compact and dense biomass with good settling properties. In the meantime, TSS concentration in the effluent dropped to 49 mg L^{-1} and the settling velocity increased to 73 m h^{-1}.

Concentrations of total and volatile suspended solids inside the reactor were also monitored over time (Figure 3.3b). During run I, no sludge was intentionally removed from the reactor, and therefore the TSS concentration reached around 8.4 g L^{-1}. With the decrease in the granules density values from runs I to III, more biomass was removed from the reactor during the effluent withdrawal phase. Moreover, in order to keep the SRT at 30 (run II) and 20 days (run III), excess

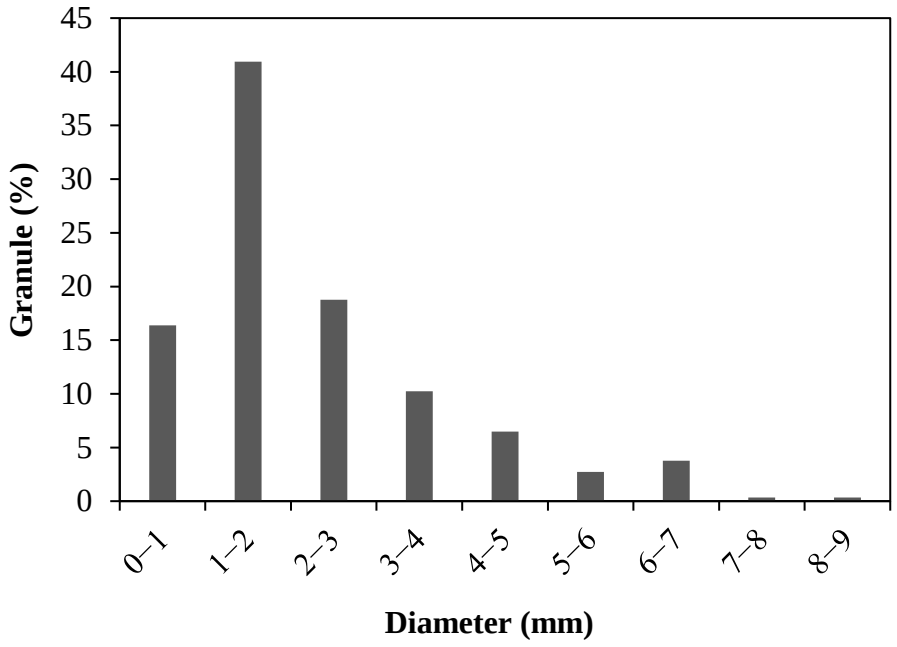
sludge had to be manually removed. Both events led to a decrease in the reactor TSS content during these experimental conditions. Average TSS and VSS concentrations corresponded, respectively, to 5.9 and 5.3 g L^{-1} in run II and 5.8 and 5.1 g L^{-1} in run III.

On the other hand, the improvement of sedimentation properties of the granular sludge led an increase in the TSS content in the reactor to around 9.6 g L^{-1} in run IV. According to Liu et al. (2010), concentrations of total biomass around 10 g L^{-1} are a good indicator of the stability of AGS-based reactors. It should be remarked that this represents an average concentration obtained over the entire phase. However, TSS content presented a dynamic behavior and tended to increase within run IV, while the VSS remained constant. This implies the accumulation of inert material (ash) in the biomass, which was observed to increase as the sludge age was gradually reduced, from 8.5% in run I to 30% in run IV, and may be related to higher polyphosphate accumulation by PAOs, as will be discussed later.

The particle diameter distribution was assessed at the beginning of run III (SRT of 20 days), and the results have shown a much dispersed distribution of the granules size (Figure 3.4a). Most of the granular particles (20%) exhibited diameters ranging between 1 and 2 mm (values commonly found for stable granules), but a considerable amount of granules (67%) were substantially big, showing diameters above 3 mm.



(a)



(b)

Figure 3.4 – Diameter distribution of the granules in a) day 138 (run III) and b) day 325 (run IV) of reactor operation.

One of the reasons for the variability in granules size is related to the filaments present in their structure, which lead to an apparent increase in their size (surface area) but not necessarily in their density (LIU, Y., LIU, 2006, WAN, C., YANG, *et al.*, 2014, FIGUEROA, VAL DEL RIO, *et al.*, 2015). Consequently, their settling properties may deteriorate over time, resulting in higher biomass washout from the SBR in the discharge phase, as observed in this study (Figure 3.3a).

Once the reactor was capable of achieving stable COD, nitrogen and phosphate removal at SRT of 15 days in run IV (as will be discussed further), granular biomass was sampled again from the reactor and subjected to optical microscopy (Figure 3.5). Under these conditions, no filaments were found in the structure of the granules. This result also coincides with the increase in the density of the granules, which returned to values between 1000 and 1010 g L^{-1} (Figure 3.1a), confirming that the absence of filamentous bacteria improves the sedimentation properties of the granules, as discussed previously.

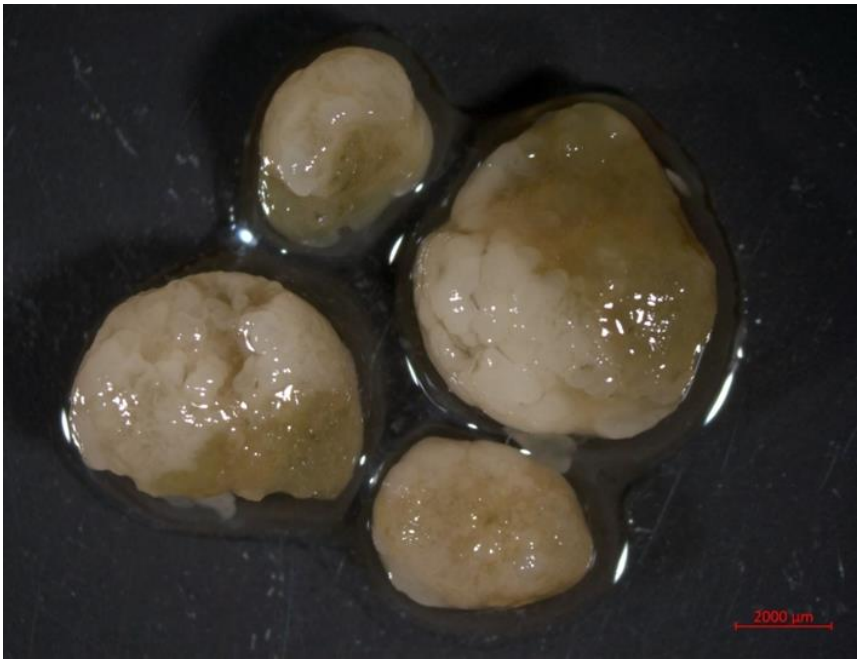


Figure 3.5 – Image of granular sludge obtained by optical microscopy after 392 days of operation (run IV).

The average diameter of the granules was also measured in the same period (Figure 3.4b), and a more homogeneous size distribution was noticed as compared to that of run III (SRT of 20 days) (Figure 3.4a). The larger fraction of the granules exhibited a diameter varying between 1 and 2 mm, a value reported by different authors as ideal for mature aerobic granules (DE KREUK, DE BRUIN, 2004, BASSIN, 2018, WANG, X. H., ZHANG, *et al.*, 2007).

It is interesting to evaluate the contribution of different forms of sludge removal at the applied SRTs. This analysis is performed by considering average values attained in each experimental run, and is displayed in Figure 3.6.

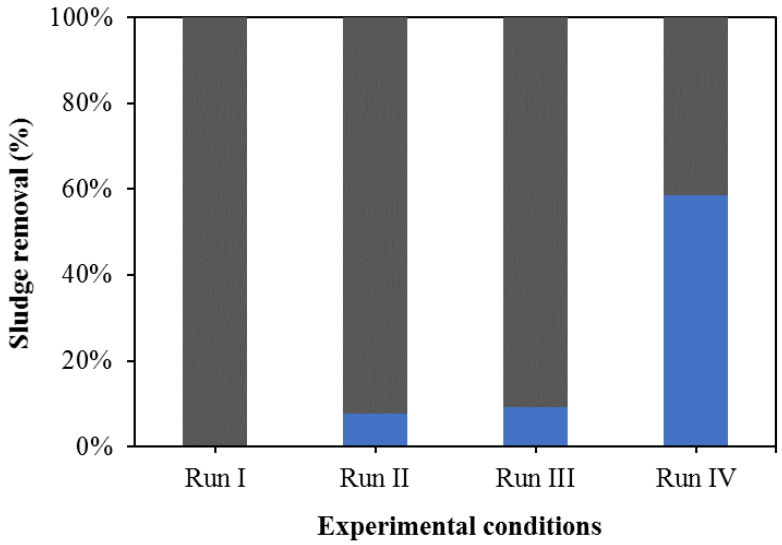


Figure 3.6 – Sludge removal by manual discharge (■) or natural washout via effluent withdrawal (■) in relation to total biomass removal throughout the different phases of reactor operation.

In run I, when the sludge age of the reactor was not intentionally controlled, sludge removal was only obtained through discharge of treated effluent containing suspended solids. When biomass started to be withdrawn to keep the sludge age at 30 days (run II), it was found that manual discharge contributed to 7% of the overall sludge removal, while the remaining (93%) was achieved via natural washout of light flocs with the final effluent. Within run III, by decreasing the sludge age to 20 days, total biomass removal by manual discharge increased to 9% and the biomass leaving the reactor as effluent solids decreased from 93% to 91%. Finally, at the lowest SRT tested in run IV (i.e., 15 days), manual sludge removal corresponded to 59% of total solids removal, exceeding that achieved by effluent withdrawal (41%).

The biomass yield coefficient (Y) was also calculated for the different operational phases of reactor. As shown in Figure 3.7, in run I, during which the sludge age was not controlled, Y was 0.24 gVSS $gCOD^{-1}$. As the SRT was reduced by manual sludge discharge, the biomass yield increased to 0.48, 0.61 and 0.68 gVSS $gCOD^{-1}$, for runs II, III and IV, respectively. Likewise, SRT reduction led an increase in the food-to-microorganism (F/M) ratio, equivalent to 0.22, 0.28, 0.29 and 0.32 kg COD kg $VSS^{-1}d^{-1}$ for runs I, II, III and IV, respectively. The F/M ratio obtained at SRT of 15 days (run IV) is within those considered favorable for stable granules with good sedimentation properties, as reported by Li; Li; Yu, (2011). The behavior shown by Y and F/M

ratio over time was expected as the decrease of SRT reduces endogenous activity and increases the amount of substrate per amount of biomass (i.e., the F/M ratio), thereby increasing sludge production. Interestingly, even though the SRT was diminished over the experimental runs, the VSS concentration tended to reach a constant value. This suggests that the increasing amount of sludge removed manually to keep sludge age at ever lower levels was compensated by the biomass growth. Such observation should be taken into account during operation of AGS reactors, to which, depending on the treatment requirements, a desired SRT can be applied with minimum influence on the volatile fraction of solids.

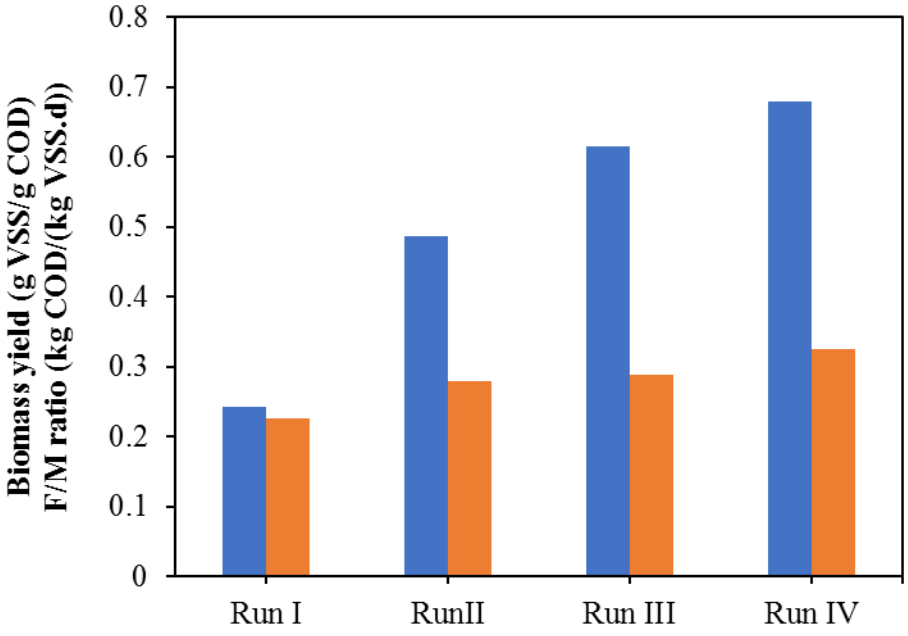


Figure 3.7 – Biomass yield (■) and F/M ratio (■) along the different operational phases in the reactor.

3.3.2. General performance of AGS reactor

The performance of the reactor during the entire operation was evaluated in terms of organic matter (COD), nitrogen and phosphorus removal. During the first 49 days of the reactor operation (run I), the sludge age was not controlled. Therefore, the SRT was influenced by the amount of

solids leaving the SBR during the effluent withdrawal phase, after a short settling period (3 min). Within this period, the sludge age varied between 47 and 61 days.

The COD profiles over the 392 days of reactor operation are displayed in Figure 3.8. The influent COD varied mostly between 300 and 400 mg L^{-1}, whereas the effluent COD ranged from 10.2 to 51.3 mg L^{-1}. The overall COD removal was generally above 95% during the entire reactor operating period, regardless of the SRT applied. From the COD mass balance, it was observed that approximately 85% of the influent organic matter was removed during the anaerobic feeding phase, when oxygen was absent. The COD remaining from the anaerobic stage (around 15%) was removed in the following aerated period.

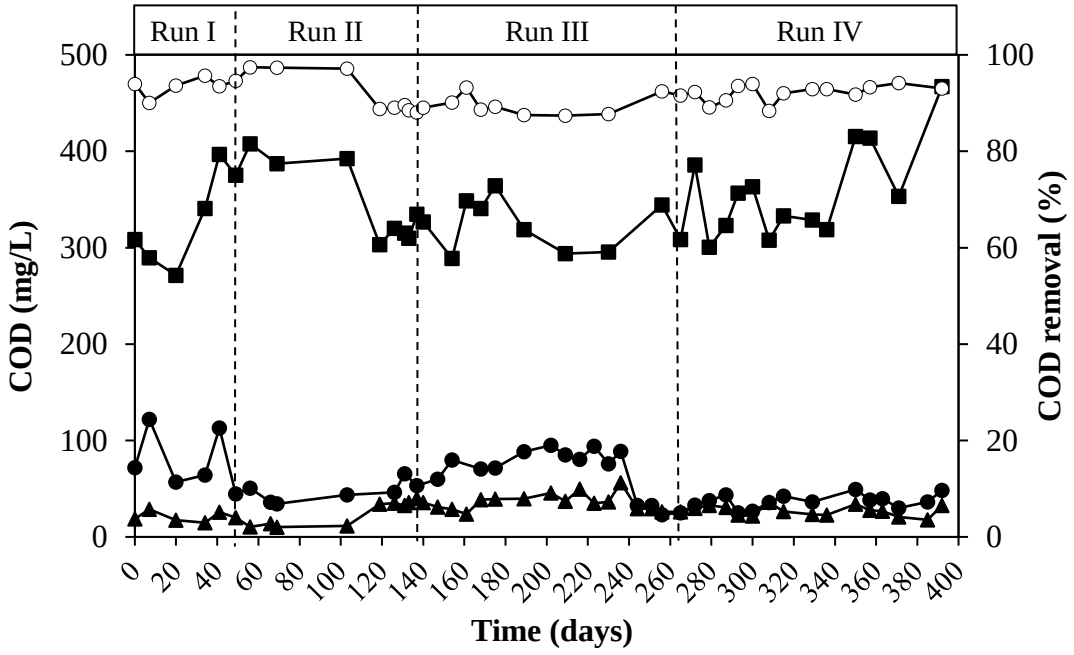


Figure 3.8 – Concentration profiles of COD in the influent (■), after anaerobic feeding (●), effluent (▲) and removal efficiency (o) during the entire operation.

Ammonium-nitrogen profiles are shown in Figure 3.9. The ammonium concentration after anaerobic feeding appear lower than the influent one due to the dilution of the incoming medium with the liquid remaining in the reactor from the previous cycle. The average influent ammonium concentration was around 55 mg L^{-1}. During the start-up period (no SRT control), ammonium removal was very unstable due to massive biomass washout, assuming values as low as 13% in

some days, for which the effluent ammonium reached 45 mgN L^{-1}. However, from day 40 onwards, practically full ammonium removal (average of 98%) was attained in the AGS reactor, demonstrating the stability of the nitrification process over time, even at the lowest SRT of 15 days applied in run IV.

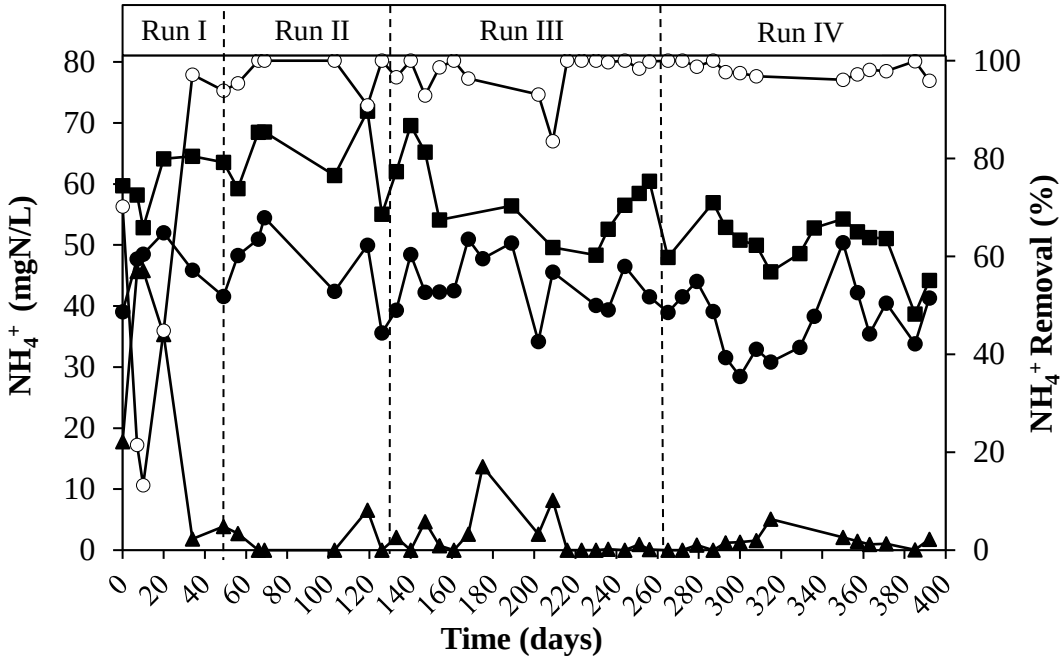


Figure 3.9 – Concentration profiles of ammonium in the influent (■), after anaerobic feeding (●), effluent (▲) and removal efficiency (o) during the entire operation.

The oxidized nitrogen compounds resulting from nitrification (i.e., nitrite and nitrate) were regularly monitored during the reactor operation for better understanding of the nitrogen conversions (Figure 3.10).

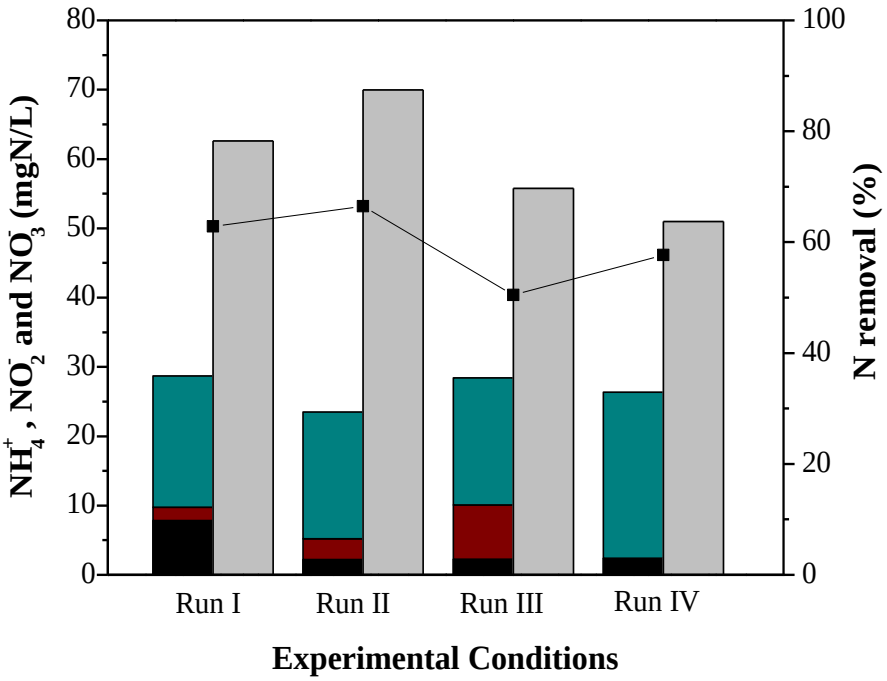


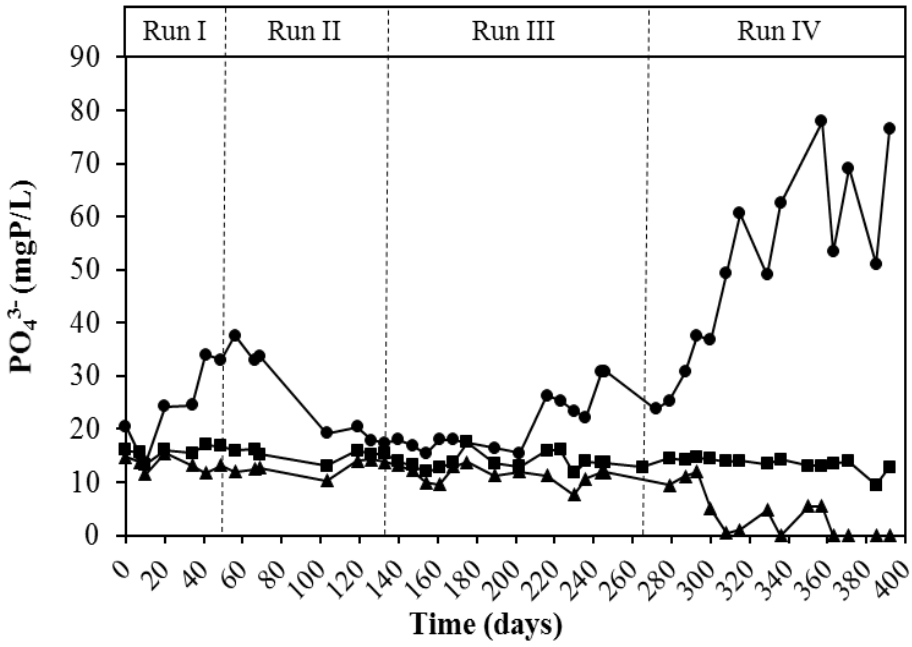
Figure 3.10 – Ammonium concentration in the influent (■), ammonium (■), nitrite (■), and nitrate (■) concentrations in the effluent, and total nitrogen removal (●) throughout the experimental runs.

In general, nitrite accounted for a small proportion of the nitrogen in the effluent, with the exception of run III, for which its average concentration reached 7.8 mg L^{-1}. Under this condition, nitrite accounted for around 30% of the overall amount of oxidized nitrogen (NO_x) species in the effluent. On the other hand, average concentrations of nitrate during the first 3 runs were around 18 mg L^{-1} and amounted up to 23.9 mg L^{-1} in run IV.

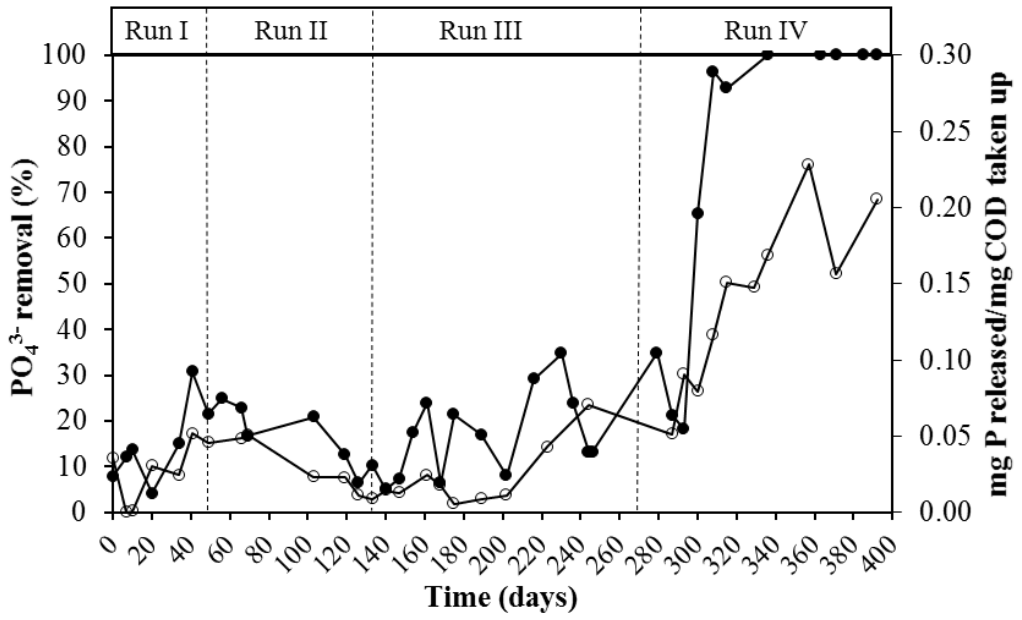
The nitrogen balance conducted for entire reactor operation period (392 days) revealed that around 90.6% of the ammonium removal was attributed to the nitrification process and 9.3% was due to biomass assimilation for growth, the latter estimated by considering a nitrogen content in the biomass of 12% (METCALF & EDDY, 2003). Considering the soluble nitrogen species in the reactor inlet and outlet (i.e., ammonium, nitrite and nitrate), a total nitrogen removal of around 58% was observed. By subtracting the total amount of nitrogen removed from that removed due to bacterial anabolism (considering average values), it was found that about 48% of the nitrogen was removed via denitrification.

DO is a key parameter for the simultaneous nitrification and denitrification (SND) process taking place in AGS systems (NANCHARAIAH, REDDY, 2018). In this study, the incomplete denitrification can be attributed to the high DO level to which the reactor was subjected (5 mg L^{-1}). Under these conditions, oxygen may penetrate deeper into the granules, enlarging their aerobic fraction and enhancing the nitrification potential. On the other hand, anoxic zone is minimized and, consequently, nitrite/nitrate reduction via denitrification is adversely affected. Therefore, reduction of DO levels would be a strategy to favor denitrifying activity and lower nitrate concentrations in the effluent (NAKANO, IWASAWA, *et al.*, 2004, PRONK, DE KREUK, *et al.*, 2015). Nevertheless, in this study, the idea was not to control the DO in a desired value to optimize SND performance, but avoid its limitation.

Despite the good performance in relation to COD and ammonium removal, the same is not valid for phosphate removal, particularly for high SRTs. As shown in Figure 3.11a, the concentration of phosphorus in the feeding was approximately 16 mg L^{-1} throughout the entire operation of the reactor, while the respective concentration in the effluent was, on average, 13.4 mg L^{-1} during the run I (SRT not controlled). This led a remarkably low phosphorus removal, amounting to a mean value of around 15% (Figure 3.11b).



(a)



(b)

Figure 3.11 – a) concentration profiles of phosphorus in the reactor inlet (■), after reactor feeding (●) and effluent (▲) during the entire operation; b) phosphorus removal efficiency (●) and phosphorus released (o) relative to the COD taken up in the anaerobic feeding period.

During the first 49 days of operation, the granules began to release phosphate during the feeding phase (around 9.4 mg P L^{-1}). Therefore, the amount of phosphate released per COD consumed started to increase (Figure 3.11b), indicating an improvement of EBPR process due to PAO activity. Nevertheless, the P removal capability was still very low, and the maximum phosphorus removal achieved during this period was only 30.7%. After day 70 (run II), the concentration of phosphate released to the medium during the feeding phase dropped sharply (P release fell to 4.5 mg P L^{-1} or 0.016 mgP released/mgCOD taken up), coinciding with the decrease in granular biomass density and formation of filamentous microorganisms. Such results indicate a reduction in PAO activity. Average phosphorus removal remained low at around 14% during run II (Figure 3.11b).

Thus, it was decided to reduce the sludge age to 20 days (run III), condition maintained for 125 days. After 63 days of operation in run III (approximately at day 200), the release of phosphate during the feeding phase started to increase (Figure 3.11b), which indicated an increase in the metabolic activity of PAO. Consequently, phosphorus removal efficiency during this phase slightly

increased, reaching an average of 16.7% and a maximum of 35%. However, these indices were still below those expected for a stable EBPR process. Therefore, the sludge age was reduced once again and maintained at 15 days for the remaining 129 days until the end of the operation (run IV).

The impact of this change was noticed after 36 days, when the phosphorus concentration in the effluent began to decrease to 3.9 mg P L^{-1} (in average), as displayed in Figure 3.11a. The release of phosphate after the feeding period (Figure 3.11b) also began to increase (in average 44.3 mg P L^{-1} or 0.14 mgP released/mgCOD taken up) and consequently the phosphorus removal increased, reaching 100% on day 308, i.e., 45 days after reduction of the sludge age to 15 days (Figure 3.11b).

The improvement of EBPR conversions during IV was accompanied by the increase in the ash content of the biomass (Figure 3.3b), which suggests an increase in the polyphosphate content due to PAO enrichment, as observed in previous study (BASSIN, KLEEREBEZEM, *et al.*, 2012a).

3.3.3. Cycle tests under normal and special conditions

Cycle tests were performed at the steady-state condition for each experimental phase in order to observe the profiles of COD, ammonium, nitrite, nitrate and phosphate over the course of the experimental cycle. The specific conversion rates are presented in Table 3.3, while the cycle tests are shown in (Figure 3.12).

Table 3.3 – Specific rates as assessed from the cycle tests conducted for each experimental condition.

Runs	**Specific NH_4^+-N uptake rate ($mgNH_4^+$-N $gVSS^{-1}$ h^{-1})**	**Specific NO_x uptake rate ($mgNO_x^-$-N $gVSS^{-1}$ h^{-1})[a]**	**Specific PO_4^{3-}-P uptake rate ($mgPO_4^{3-}$-P $gVSS^{-1}$ h^{-1})**
Run I	7.84	1.02	4.6
Run II	8.64	5.8	2.4
Run III	6.59	2.97	10.7
Run IV	6.95	2.74	11.4

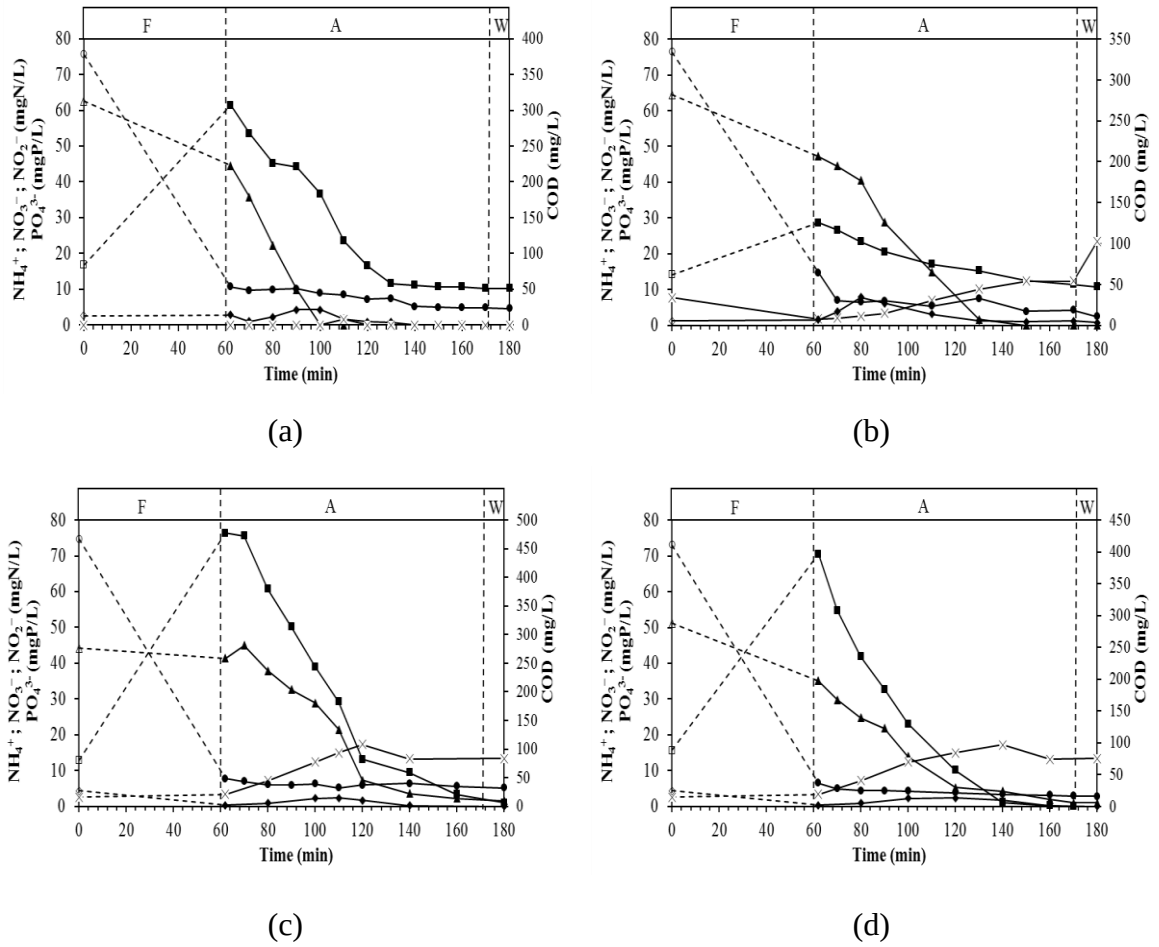


Figure 3.12 – Cycle tests performed along the different operational phases: a) day 2 (run I); b) day 78 (run II); c) day 261 (run III); d) day 392 (run IV). The COD profiles (●), ammonium (▲), phosphorus (■), nitrite (♦) and nitrate (×) are shown. The symbols (o, Δ, □) represent the affluent concentrations of COD, ammonium and phosphorus, respectively. The letters F, A and W refer to anaerobic feeding, aerated phase and effluent withdrawal phase, respectively. No sample was collected during the anaerobic feeding period (non-mixed reactor), so the dashed line is just an indication of the tendency to increase or decrease of a certain compound.

As it can be observed, the removal of COD and ammonium was almost complete in all runs, corroborating with the results obtained during daily monitoring of the reactor, as discussed previously. Most of the incoming organic matter (in the form of acetate) was consumed by the microorganisms during the anaerobic feeding phase and the remaining was biodegraded during the aerated phase, thus, the overall COD removal efficiency was higher than 93%. As regards to ammonium profiles, its concentration decreased linearly over the aeration period.

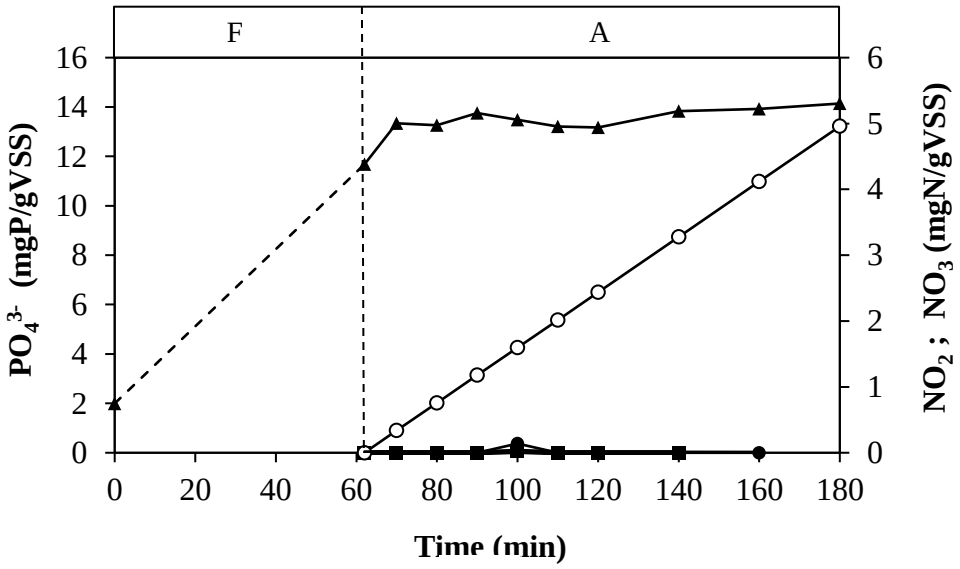
Biomass specific nitrification rates were determined from the cycle tests for all experimental runs. Highest values were found for runs I and II (7.8 and 8.6 mgNH_4^+-N $gVSS^{-1}$ h^{-1}), respectively, which coincides with the higher sludge ages applied to the reactor. With the reduction of SRT to 20 and 15 days during runs III and IV, the specific nitrification rates decreased to 6.6 and 6.9 mgNH_4^+-N $gVSS^{-1}$ h^{-1}, respectively. Nevertheless, taking into the amount of biomass within the reactor, such ammonium-oxidizing activity was sufficient to fully nitrify the ammonium present, so that this substrate was no longer detected after 130 min from the start of the cycle.

Nitrite build-up was observed over the cycle, and its maximum concentration in runs I, II III and IV was, respectively, 4.2, 7.8, 2.4 and 2.4 mgN L^{-1}. Nevertheless, during the aerated period, nitrite was further oxidized to nitrate or reduced via denitrification, given that its effluent concentrations were always below 1 mgN L^{-1}. On the other hand, nitrate concentrations tended to increase gradually along the aeration cycle, as nitrification proceeded. However, nitrate formation did not occur at the same proportion to the ammonium oxidation, indicating simultaneous ammonium oxidation (by nitrification) and nitrate reduction (by denitrification) taking place in the aerobic and anoxic layers of the granules, respectively. The specific denitrification rates estimated from the cycle experiments for runs I, II, III and IV were 1.02, 5.8, 2.97 and 2.74 mgNO_x^-N $gVSS^{-1}$ h^{-1}, respectively. The highest value found for run II can be associated with the larger size of the granules, a favourable condition for enhancing denitrification, as discussed previously.

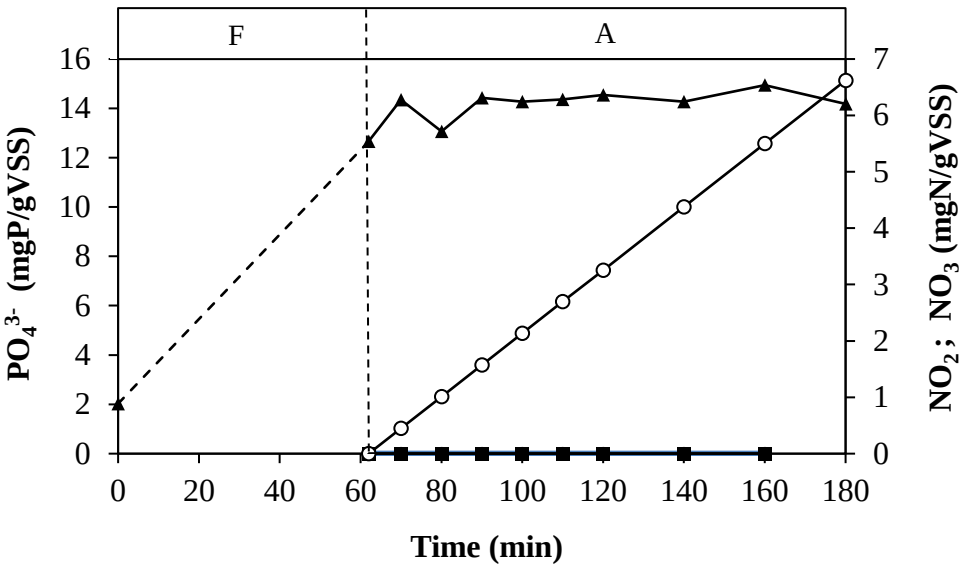
An interesting observation can be made regarding the profile of the nitrogen compounds over the aeration phase. Although the carbon source (as PHA) stored intracellularly by DPAOs or denitrifying GAOs (DGAOs) could be used as electron donor for denitrification throughout the SBR cycle, this was observed as long as ammonium was still present in the bulk. Under these conditions, nitrate resulting from nitrification was simultaneously reduced via denitrification. However, once ammonium has been fully oxidized, nitrate concentrations remained fairly constant and it was not further denitrified. This is possible associated with DO dynamics within the granular structure. In the later situation, the nitrogenous demand posed by nitrifiers in the outer layers of granular biomass could prevent further DO penetration within the granules, allowing denitrification to take place in the inner zones. Once ammonium was completely nitrified, there was no oxygen consumption by ammonium-oxidizing activity, so further DO penetration could have occurred, hampering the establishment of anoxic conditions required for denitrification.

On the other hand, phosphate release and uptake profiles fluctuated a lot and P removal efficiency was largely related to the prevailing SRT. During the cycle test carried out in run I, anaerobic phosphate released was quite high (61 mg L^{-1}), indicating that the influent carbon source was anaerobically taken up by PAO. However, the residual phosphate in the effluent (10.2 mg P L^{-1}) remained high. Throughout run II, the COD uptake under anaerobic conditions remained high but the P release drastically decreased to 28 mg P L^{-1}. This result suggests that other flanking organisms, such as GAOs, were converting the available carbon source into PHA anaerobically. As the sludge age was reduced in runs III and IV, both the release of phosphorus in the anaerobic phase and uptake in the aerated phase increased, indicating that SRT reduction favored the metabolic activity of PAO. Indeed, the specific phosphate uptake rates tended to increase as the sludge age was diminished, reaching a maximum of 11.4 mgPO_4^{3-}-P $gVSS^{-1}$ h^{-1} in run IV.

During run III, when the phosphorus removal capacity has been improved, cycle tests under anoxic conditions were also performed, as explained in section 3.2.3. The intention was to assess the phosphate uptake potential with nitrate as electron acceptor, i.e., coupled to denitrification (denitrifying dephosphatation). Two experiments were carried out, one dosing nitrate according to the denitrification rate estimated from a normal cycle test (Figure 3.13a), and other adding this compound at twice as the regular denitrification rate (Figure 3.13b).



(a)



(b)

Figure 3.13 – Cycle tests carried out under anoxic conditions with nitrate being dosed according to the denitrification rate (a) and at twice the denitrification rate observed under normal operating conditions (b). Phosphorous (▲), nitrite (■), nitrate (●) and added nitrate (○) given in mass (mg) per unit of volatile solids (gVSS). No sample was collected during the anaerobic feeding period (non-mixed reactor), so the dashed line is just an indication of phosphorus profile tendency.

Phosphate release took place anaerobically, as normally obtained. However, the replacement of oxygen by nitrate as electron acceptor during the phase when the reactor was continuously sparged with nitrogen gas was detrimental to phosphate uptake. Indeed, no phosphate removal was observed in such tests, regardless of the dosage that this compound was added. Moreover, almost complete nitrate reduction was obtained, even in the test where it was dosed as twice as the denitrification rate. This implies that the reactor was capable of reducing a higher amount of nitrate than that achieved under normal operating conditions at high DO (for which nitrate is always detected in the effluent), as long as oxygen is kept at the lowest possible levels (as in the case of the anoxic cycle tests).

The cycle tests carried out anoxically may also provide insights into the main responsible for the denitrification process taking place within the granules. Since there was no external COD available after the feeding phase, only DPAOs and DGAOs could carry out denitrification, as these

organisms could use their intracellularly accumulated PHA as electron donor for NO_x reduction. However, by showing that there was no phosphate uptake coupled to nitrate reduction, the cycle tests carried out anoxically suggest that the denitrifying GAOs were the key players in the denitrification process under normal reactor operating conditions.

3.3.4. FISH analysis

FISH analysis was conducted in granular sludge samples from runs I (uncontrolled SRT) and IV (SRT of 15 days) to track the dynamics of important microbial functional groups (PAO, GAO, AOB and NOB) for the AGS reactor under two completely different scenarios in terms of SRT. The average values from the quantification analysis of all groups are displayed in Figure 3.14, while the images acquired are presented in Figure 3.15 and Figure 3.16.

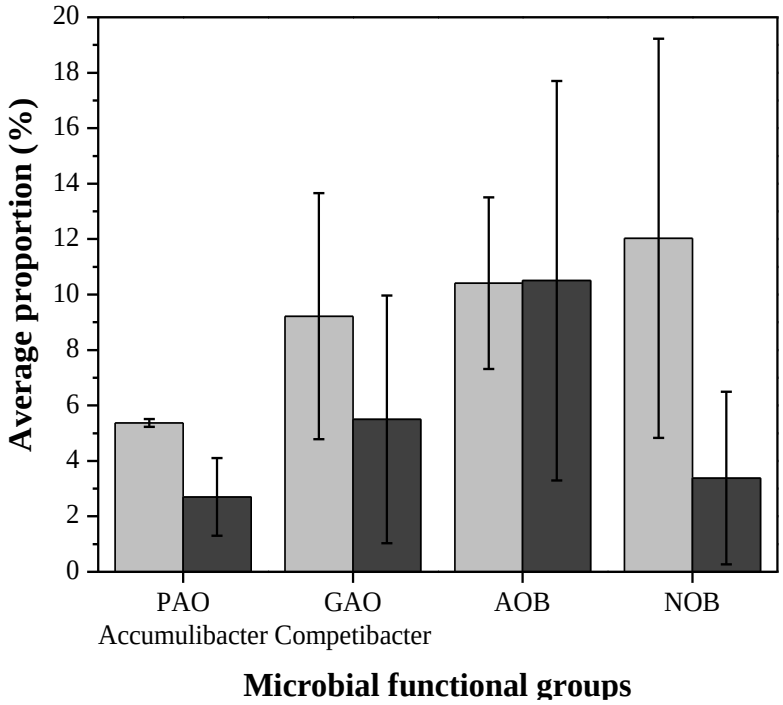
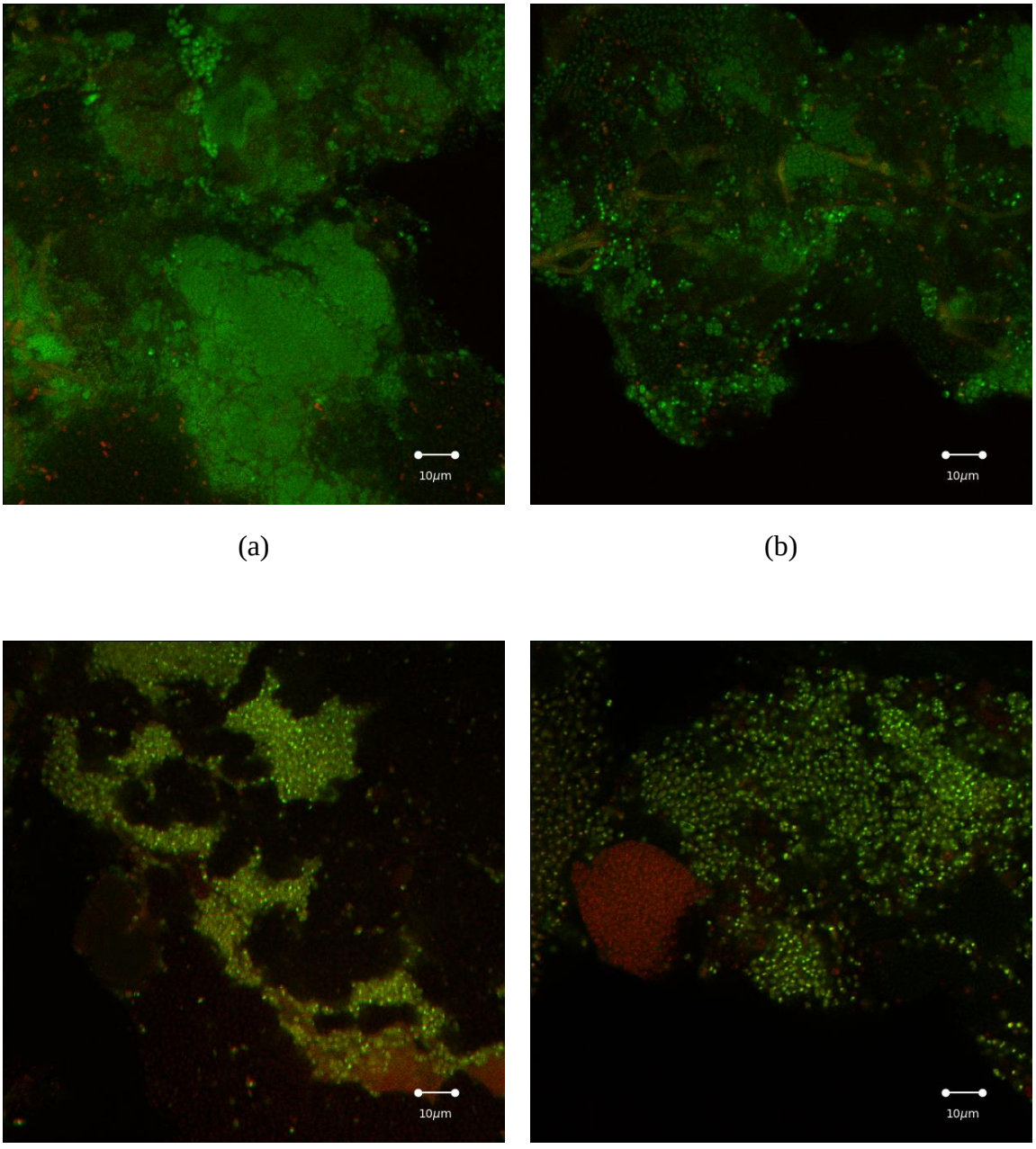


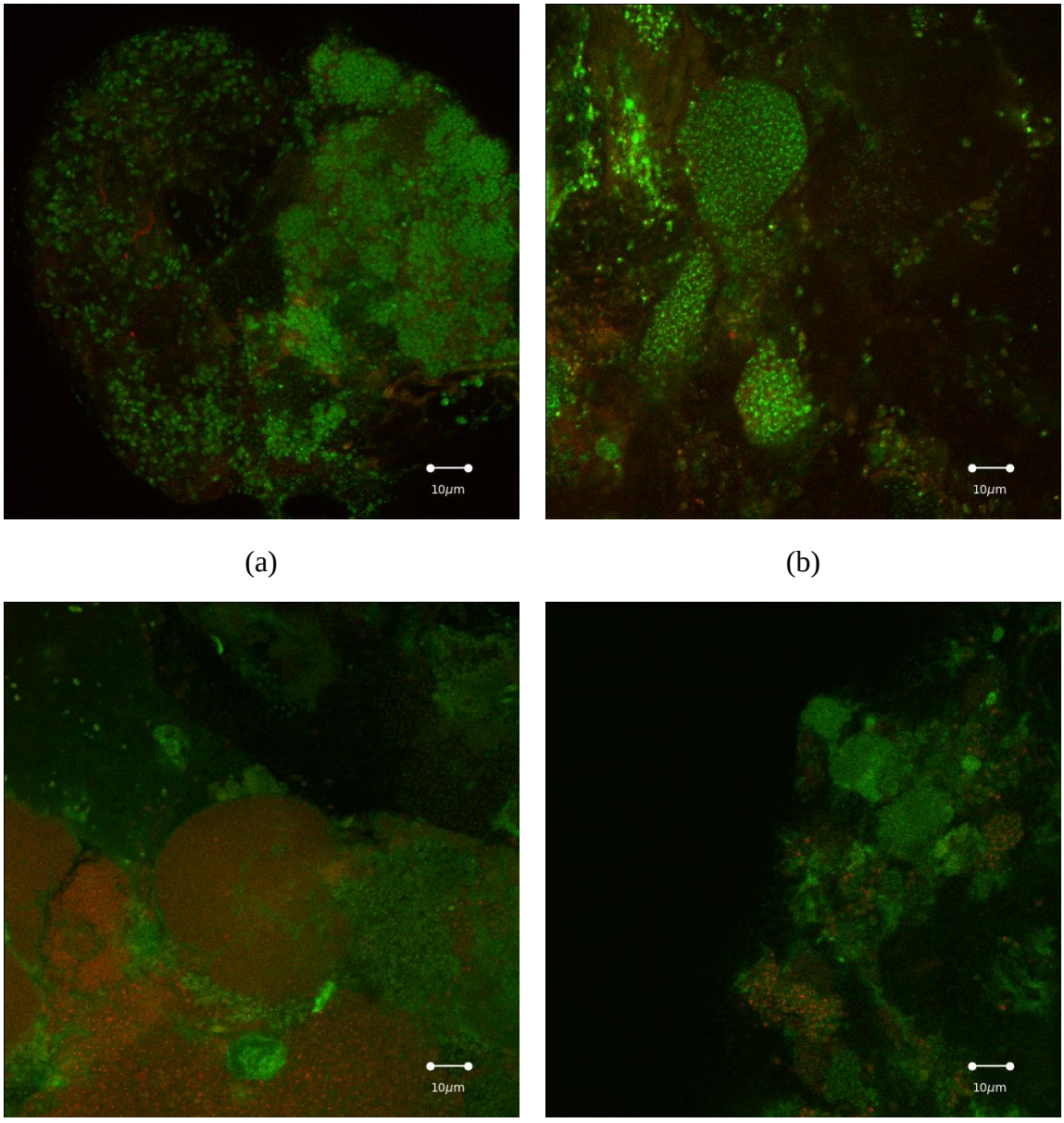
Figure 3.14 – Microbial functional groups present in the granular sludge sample identified by FISH analysis during the run I (■) and run IV (■) of reactor operation.



(a) (b)

(c) (d)

Figure 3.15 – Microscopic FISH images of fixed granule samples showing targed bacterial communities in fluorescent dye (red) within total bacterial community (green): a) Accumulibacter-related PAOs (run I), b) Accumulibacter-related PAOs (run IV), c) Competibacter-related GAOs (run I), d) Competibacter-related GAOs (run IV).



(a) (b)

(c) (d)

Figure 3.16 – Microscopic FISH images of fixed granule samples showing targed bacterial communities in fluorescent dye (red) within total bacterial community (green): (a) AOB (run I), (b) AOB (run IV), (c) NOB (run I) and (d) NOB (run IV).

Specific probes for *Candidatus* Accumulibacter phosphatis (commonly referred to as Accumulibacter) were used to target PAOs, as this particular genus is considered to be the major PAOs in EBPR systems (CROCETTI, HUGENHOLTZ, *et al.*, 2000). For identification of GAOs, probes targeting *Candidatus* Competibacter phosphatis (also designated as Competibacter) were used, given their relative importance as competitor for PAOs in biological phosphate removal systems (CROCETTI, BANFIELD, *et al.*, 2002).

The percentage of Accumulibacter-related PAOs within the total bacterial community of the reactor in run I was 5.5%, while the corresponding value for run IV was 2.7%. In principle, it was expected a higher contribution of Accumulibacter in the later experimental condition, when better EBPR performance was observed. However, this was not the case. It should be clear that the probes used do not target the entire PAOs community, but only Accumulibacter-related organisms. Therefore, other organisms capable of performing the typical EBPR conversions (NIELSEN, MIELCZAREK, *et al.*, 2010, NGUYEN, LE, *et al.*, 2011, MIELCZAREK, NGUYEN, *et al.*, 2013), not detected by FISH probes used this study, may have been present in the granular biomass. Moreover, it should also be noted that the proportion of Competibacter-related GAOs within the overall bacterial community also diminished with SRT reduction, from 9.2% (run I) to 5.2% (run IV), result that can be associated with the better biodephosphatation performance observed at lower SRT.

Actually, the decrease of the relative importance of these organisms is convenient for achieving high and stable biological phosphate removal, and they compete with PAO for the substrate under anaerobic conditions but cannot accumulate phosphate, being therefore regarded as undesired organisms in EBPR systems (NIELSEN, MIELCZAREK, *et al.*, 2010, MIELCZAREK, NGUYEN, *et al.*, 2013).

With respect to the AOB community in the reactor, it remained practically the same from run I to run IV, with average frequency of 10.5% for both regimes. Interestingly, the proportion of NOB was slightly higher than that of AOB in run I (12%), but it drastically decreased in run IV (3.8%). Usually, the amount of AOB is higher than that of NOB, since the product of the first (nitrite) is the substrate for the second. Moreover, AOB obtain more energy per mole of ammonium oxidized than the NOB does by performing nitrite oxidation (FREITAG, RUDERT, *et al.*, 1987).

However, a disproportion of these two bacteria was already reported in AGS (WINKLER, BASSIN, KLEEREBEZEM, SOROKIN, *et al.*, 2012), which consist of multilayer clusters of

organisms where many simultaneous conversions take place. In this context, NOB may have access not only to the nitrite resulting from autotrophic ammonium oxidation by AOB, but also from nitrate reduction by denitrifiers. The additional supply of nitrite by this process, referred to as "nitrite loop", may boost the growth of NOB whose relative importance may surpass that shown by the AOB (WINKLER, BASSIN, KLEEREBEZEM, SOROKIN, *et al.*, 2012).

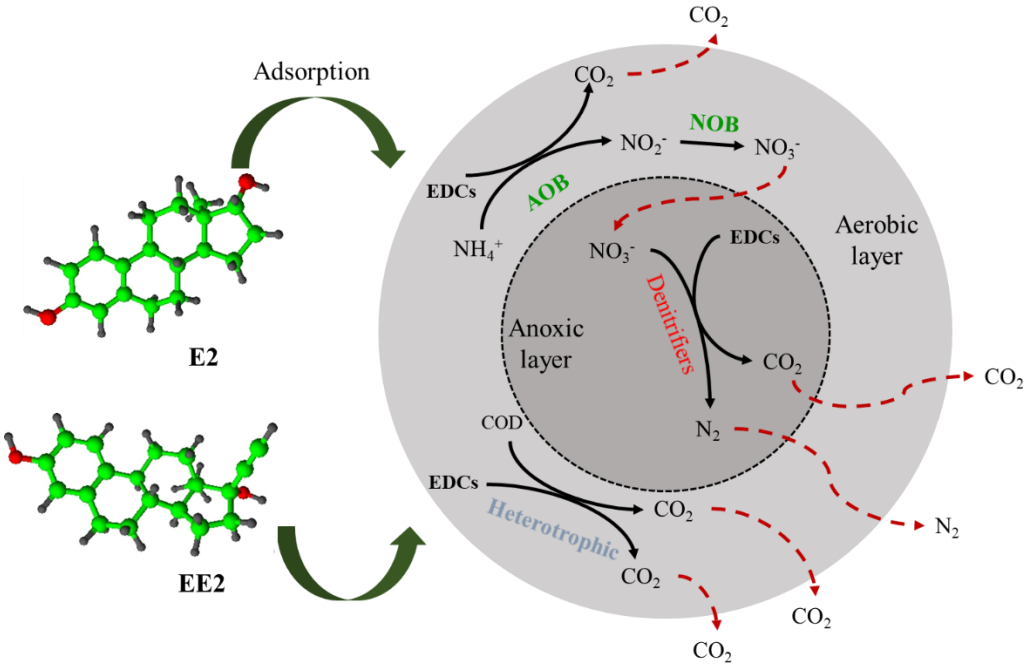
3.4. Conclusions

The effect of sludge retention time (SRT) on the stability and performance of aerobic granular sludge reactor was assessed in this study. The results have shown that operating the reactor without sludge age control, high COD and ammonium removal was obtained, but phosphate removal was considerably low (15%). At SRT of 30 days, no improvement with respect to the EBPR process was achieved. Besides, proliferation of filamentous microorganisms in the granules surface was observed, adversely affecting their physical properties and stability. At lower SRTs (20 and 15 days), phosphorus removal efficiencies improved, reaching of 35% and 100% respectively. In the latter condition, the amount of P released per COD taken up and the specific phosphate uptake rates reached maximum values (0.14 mgP $mgCOD^{-1}$ and 11.4 $mgPO_4^{3-}$-P $gVSS^{-1}$ h^{-1}, respectively), while the removal of COD, ammonium and total nitrogen reached 93%, 97% and 58%, respectively.

4. 17β-Estradiol and 17α-Ethinylestradiol Removal in an Aerobic Granular Sludge Sequencing Batch Reactor Accomplishing Simultaneous COD, Nitrogen and Phosphorus Removal: Evaluating Estrogenic Activity and Estrogens Fate

Article submitted to the Water Research on: 22/07/2020

4.1. Graphical abstract



4.2. Introduction

The growing development of synthetic products, such as pharmaceuticals, cleaning agents and personal care products (PCPs) has boosted the presence of polluting substances in water-receiving environments. These substances, present in very low concentrations (in order of micrograms and nanograms per litre) in the environment, are also known as Contaminants of Emerging Concern (CECs), has been drawing the attention of the environmentalists and researchers due to their adverse effect on aquatic organisms and even in humans (BILA, DEZOTTI, 2007).

Conventional treatment processes employed in sewage treatment plants are not effective enough to remove this type of substances, and, therefore, they may inevitably reach the aquatic environments (MARGOT, LOCHMATTER, *et al.*, 2016). Endocrine disrupting chemicals (EDCs), which are exogenous substances that can affect health in an intact organism or its progeny causing changes in the endocrine functions, are among these contaminants (BALEST, LOPEZ, *et al.*, 2008). Natural and synthetic estrogens are considered the main EDCs due to their high estrogenic potency capable of causing

changes in organisms even at low concentrations (ROUTLEDGE, SHEAHAN, *et al.*, 1998, DE MES, ZEEMAN, *et al.*, 2005).

Natural estrogens are steroid hormones produced primarily by the ovaries and adrenal glands, being responsible for the development of the female reproductive system (SHIMADA, MITAMURA, *et al.*, 2001). The hormone 17β-estradiol (E2) fall within this category. On the other hand, synthetic estrogens (e.g., 17α-ethinylestradiol – EE2) are used as contraceptives and in hormone therapy treatments (DE MES, ZEEMAN, *et al.*, 2005). Both are excreted by the human body through urine and faeces either in their conjugated form, as glucuronides or sulphates, or in their most biologically active free form (BALEST, LOPEZ, *et al.*, 2008, LIU, Z. H., LU, *et al.*, 2015). Some studies have reported the estrogen concentrations in sanitary sewage samples varying from the equipment detection limits up to 21 ng L^{-1} for E2 and 5 ng L^{-1} for EE2 (TERNES, KRECKEL, *et al.*, 1999).

Although the analytical techniques for detecting EDCs may provide useful insights into their presence or absence in a certain sample, complimentary *in vitro* and *in vivo* assays are commonly used to evaluate the potential effects of estrogenic substances on target organisms and avoid improper release of wastewater into water bodies. *In vivo* assays usually involve the use of animals or plants, however the use of these experiments may give rise to ethical concern (DO NASCIMENTO, SANTOS, *et al.*, 2018). In contrast, besides proving accurate results, *in vitro* assays are easier to implement since they rely on the use of microorganisms, cells or biomolecules in a laboratory environment. One example of a useful, efficient, fast and reliable *in vitro* method to assess the estrogenic activity in a variety of matrices is the yeast estrogen screen (YES) bioassay (DIAS, GOMES, *et al.*, 2015). This test is able to identify the estrogenic potential of many substances present, for instance, in wastewater, as well as the by-products resulting from their degradation.

The removal endocrine disrupters and their associated estrogenic activity can be accomplished by different means, including biological (CLARA, STRENN, *et al.*, 2004, WEBER, S., LEUSCHNER, *et al.*, 2005, PETRIE, MCADAM, *et al.*, 2014, LUO, GUO, *et al.*, 2014, LUO, JIANG, *et al.*, 2015), electrochemical (CONG, IWAYA, *et al.*, 2014, HE, HUANG, *et al.*, 2017, HUA, HE, *et al.*, 2019) and advanced oxidation processes, such as ozonation (BILA, MONTALVÃO, *et al.*, 2007, NAKADA, SHINOHARA, *et al.*, 2007, MANIERO, BILA, *et al.*, 2008, LARCHER, DELBÈS, *et al.*, 2012), and photocatalysis (BELGIORNO, RIZZO, *et al.*, 2007, BENOTTI, STANFORD, *et al.*,

2009, CASTELLANOS, BASSIN, *et al.*, 2020). Despite the existence of several alternatives that allow the removal of these micropollutants, biological treatment processes are environmentally friendly solutions with lower costs as compared to other physicochemical counterparts. Besides, they are usually considered to be the heart of municipal wastewater treatment plants, being very effective for the removal of conventional pollutants, such as organic matter (often expressed as chemical oxygen demand – COD) and nutrients (nitrogen and phosphorus). On the other hand, the removal of CECs by biological means is more troublesome and deserves special attention.

Many previous studies have investigated the biological removal of EDCs by means of activated sludge-based processes (CLARA, STRENN, *et al.*, 2004, WEBER, S., LEUSCHNER, *et al.*, 2005, PETRIE, MCADAM, *et al.*, 2014), membrane bioreactors (DE GUSSEME, PYCKE, *et al.*, 2009, CLOUZOT, DOUMENQ, *et al.*, 2010), and biofilm systems, as moving-bed biofilm reactors and their hybrid configurations (PIEPER, ROTARD, 2011, LUO, GUO, *et al.*, 2014, LUO, JIANG, *et al.*, 2015, JIANG, Q., NGO, *et al.*, 2018). However, the complexity inherent to biological processes, their strong dependency on environmental factors (e.g., temperature, pH, dissolved oxygen) and reactor operating conditions (e.g., hydraulic retention time, solids retention time and food-microorganism ratio), along with the relatively low concentrations that EDCs are found in comparison to other pollutants, may hinder the biodegradation of these compounds (LI, F., YUASA, *et al.*, 2005, KOH, CHIU, *et al.*, 2008, TRAN, URASE, *et al.*, 2013, LUO, GUO, *et al.*, 2014, FALÅS, WICK, *et al.*, 2016, JIANG, Q., NGO, *et al.*, 2018).

In recent years, an innovative biological treatment process referred to as aerobic granular sludge (AGS) has been attracted the attention of environmental engineers and researchers due to its intrinsic characteristics (BASSIN, 2018). AGS process is based on self-aggregation of bacteria in the form of granules, in which multiple redox layers (aerobic and anoxic/anaerobic) are formed and a wide range of microbial functional groups coexist (ADAV, LEE, *et al.*, 2008). This may allow simultaneous conversions (COD, nitrogen and phosphate removal) to take place in a single reactor unit, usually operated as a sequencing batch reactor (SBR), reducing operating costs and installation space (DE KREUK, DE BRUIN, 2004, SEVIOUR, PIJUAN, *et al.*, 2008, BASSIN, KLEEREBEZEM, *et al.*, 2012a). The feeding of the reactor under anaerobic conditions enable the enrichment of polyphosphate accumulating organisms (PAOs) in the sludge bacterial community, allowing biological dephosphatation by the so-called Enhanced

Biological Phosphorus Removal (EBPR) process, while simultaneous nitrification/denitrification is made possible by providing aeration in a subsequent phase of the SBR operation (BASSIN, KLEEREBEZEM, *et al.*, 2012a).

Despite these attractive features of AGS technology, few works has addressed the performance of aerobic granules on the removal of micropollutants (LIU, Y., WANG, *et al.*, 2008, MOREIRA, I. S., AMORIM, *et al.*, 2015, ZHAO, B. H., CHEN, *et al.*, 2017). Furthermore, there is limited information regarding the biodegradation of the endocrine disrupting compounds in this type of reactors (BALEST, LOPEZ, *et al.*, 2008, ZHENG, HE, *et al.*, 2015, MARGOT, LOCHMATTER, *et al.*, 2016), a subject that needs further exploration. Therefore, in this contribution, the performance of an AGS reactor in the removal of two estrogenic compounds, namely E2 and EE2, from a synthetic wastewater simulating domestic sewage was addressed, tracking their fate over the treatment, while monitoring the reactor performance in terms of COD, N and P removal. The micropollutants conversions under different redox conditions and their effect on the specific bacterial functional groups were also assessed. YES bioassays were also conducted to assess estrogenic activity of effluent samples, complementing the results obtained during reactor operation. From the profile of the analysed micropollutants over the SBR cycle and their quantification in both biomass and liquid samples, a possible sequence of steps for their removal, including the key microbial players, was proposed.

4.3. Materials and Methods

4.3.1. Experimental set-up and reactor operation

Experiments were carried out in a lab-scale bubble column aerobic granular sludge (AGS) sequencing batch reactor (SBR). The reactor, made of plexiglass, had a useful volume of 1.5 L, an internal diameter of 5 cm and useful height of 79 cm. A Programmable Logic Controller (PLC) coupled with VersaPro data acquisition software was used to control each operating phase of the SBR. The reactor was subjected to alternating anaerobic and aerobic conditions to allow simultaneous nitrogen and phosphate removal. For this purpose, the SBR cycle started with a feeding phase performed under anaerobic conditions for 60 min during which 0.95 L of the synthetic influent (whose composition is described next) was fed from the bottom of the column in a plug-flow regime through the settled biomass bed. Then, an aerated phase took place

over 112 min, within which aeration was supplied through an air diffuser placed at the bottom of the column at a flow rate of 2.2 L min^{-1}, ensuring complete mixing of the reactor content. After this period, aeration was interrupted and the biomass was allowed to settle for only 3 min, making it possible to keep fast-settling granules within the reactor while causing selective washout of poor settling flocculent sludge. Finally, the treated effluent was withdrawn by an output port located 29 cm from the reactor bottom, resulting in a volume exchange ratio of 63% and a hydraulic retention time (HRT) of 4.7 h. The reactor scheme is presented in Figure 4.1.

The sludge age was controlled by periodic manual removal of the biomass from the reactor at the end of the cycle (after effluent withdrawal). This parameter was kept invariable at 15 days, a condition reported to be favourable for the granular biomass settling properties and for COD and nutrients removal (WINKLER, KLEEREBEZEM, *et al.*, 2012, ZHANG, C., LI, *et al.*, 2016). Average dissolved oxygen (DO) concentration within the aerated phase was kept at 5 mg L^{-1}, but it varied throughout the SBR cycle between 0.1 to 6.6 mg L^{-1}. The reactor was maintained at room temperature (20 ± 3 ºC) during the entire operation

Initially, in a first stage (Stage I), no EDCs were added to the reactor influent. During this period, designated as control experiment, the reactor has already been running with pre-formed granules, being fed with a synthetic wastewater consisting of two solutions (A and B), whose composition is detailed in Table 4.1. A volume of 158.3 mL of each solution was mixed with 633.2 mL of tap water to achieve influent concentrations of 50 mg NH_4^+-N L^{-1}, 16 mg PO_4^{3-}- P L^{-1} and 400 mg L^{-1} of COD, a typical composition of domestic sewage. A trace element solution (VISHNIAC, SANTER, 1957) was added to solution B in a proportion of 5 mL L^{-1} to favour the growth of microorganisms.

Table 4.1 – Solutions used to prepare the synthetic influent fed to the AGS-SBR.

Solution A (mM)		**Solution B (mM)**	
$NaCH_3COO\ 3H_2O$	31.5	NH_4Cl	21.3
$MgSO_4\ 7H_2O$	1.8	K_2HPO_4	2.1
KCl	2.3	KH_2PO_4	1.1
$CaCl_2$	3.3	Trace element solution	

Subsequently, in a second stage (Stage II), estrogens (namely E2 and EE2, both acquired from Sigma-Aldrich, 98% purity) were added to the reactor influent. For this

purpose, a solution containing both estrogens (referred to as EDC solution), stored in a third tank (E) kept under refrigeration (4 ºC) to prevent degradation which would potentially occur at room temperature, was added to the reactor feeding. A volume of 158.3 mL of EDC solution was mixed with 158.3 mL of solutions A and B and 479 mL of tap water to maintain the same influent COD, N and P concentrations of Stage I and achieve a concentration of 20 µg L^{-1} of both E2 and EE2 in the reactor inlet.

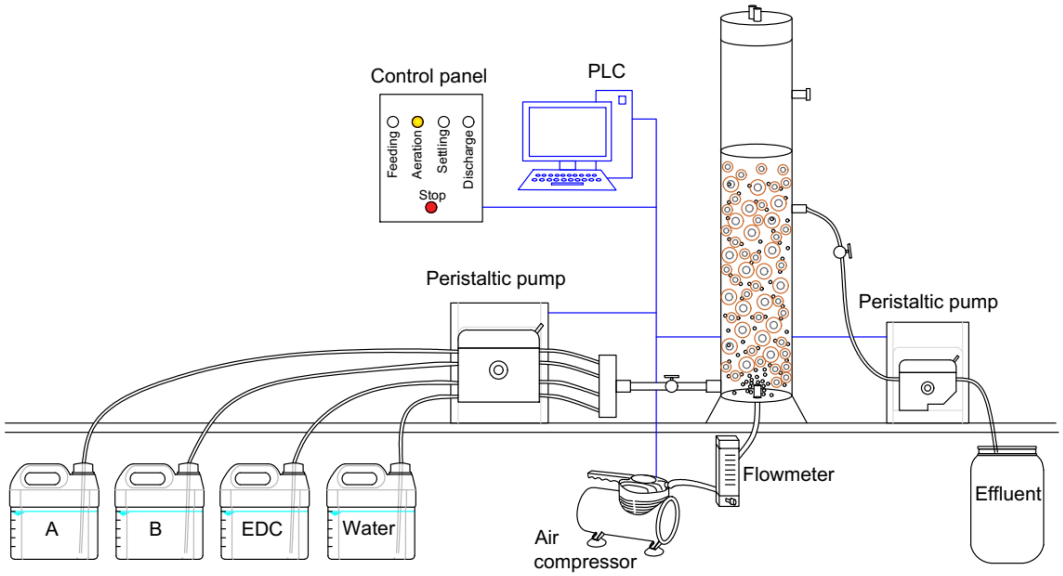


Figure 4.1 – Experimental system composed of the AGS-SBR and the other units (peristaltic pumps, influent and effluent storage vessels, air compressor, Programmable Logic Controller (PLC) and associated software/computer) that integrate the treatment process. The composition of the solutions A, B, EDCs (kept under refrigeration at 4 °C) is further described in the text.

4.3.2. Cycle tests to assess specific conversion rates

Typical cycle tests were carried out when steady-state conditions were achieved in both Stages I and II. Liquid samples were collected every 10 to 20 min only over the aerated phase of the SBR, during which the reactor content was homogenized. The first sample was collected 2 min after aerated phase started (i.e., 62 min of the overall cycle) to allow enough mixture. COD, ammonium, nitrite, nitrate and phosphate concentration profiles were plotted over the 180 min of the cycle. E2 and EE2 concentrations were also tracked over the cycle in Stage II. Ammonium and phosphate specific uptake rates were determined by linear regression of their concentrations divided by the volatile suspended

solids (VSS) concentration in the reactor. The denitrification rate was calculated based on the difference between the ammonium uptake rate and the nitrite and nitrate production rate, as suggested by Bassin, et al. (2012c).

4.3.3. Biomass physical properties

The average size of the granules was measured using Image J analyzer software. The granules density was measured with a pycnometer following the methodology reported by Winkler et al. (2012). The sludge volume index (SVI), that indicates the volume occupied per mass unit of sludge (mL $gTSS^{-1}$) after settling for 5 min (SVI_5) and 30 min (SVI_{30}), was determined according to the methodology proposed by Schwarzenbeck et al. (2004) and (APHA, 2005).

Changes in the granules structure during the estrogens addition to the reactor in Stage II were monitored by means of scanning electron microscopy (SEM) analysis. Sample preparation was carried out as follows: first the sample was washed two times with a PBS solution (pH = 7.2) during 5 min each. Then, fixation was made with Karnovsky fixative solution (paraformaldehyde 16%, glutaraldehyde 25%, cacodylate buffer 0.2 M and Milli-Q water) for 1 h. Subsequently, the sample was washed three times with cacodylate buffer 0.1 M (10 min each). A post-fixation was performed with OsO_4 2% and FeCNK 2.5% solution (1:1) in cacodylate buffer 0.1 M for 1 h, followed by four washing steps with cacodylate buffer 0.1 M (10 min each). The sample was gradually dehydrated by successive immersions of 10 min each in ethanol solutions of gradually increased concentration (30%, 50%, 70%, 90% and 100%) and then dried in a critical point drying equipment (BAL-TEC CPD 030, Liechtenstein). After the drying procedure, the samples were placed in aluminum stubs previously covered with carbon adhesive tape for microscopy and taken to a metallizer (BALTEC SCD 050 Sputter Coater, Liechtenstein) for coating with gold. Finally, the sample was observed in a scanning electron microscope (Jeol SEM 5310).

4.3.4. Analytical procedures

Chemical oxygen demand (COD), ammonium (NH_4^+-N) and phosphate (PO_4^{-3}–P) concentrations were measured by standard colorimetric methods, according to APHA (2005). Nitrite (NO_2^--N) and nitrate (NO_3^- -N) concentrations were detected by injection

of previously filtered samples (0.45 µm acetate filters) in an ion chromatograph (Metrohm Professional 850 IC). Total suspended solids (TSS) and volatile suspended solids (VSS) in the effluent were measured using thermogravimetric methods (APHA, 2005). Biomass concentration (TSS and VSS) inside the reactor was determined according to Bassin et al. (2012a). For the quantification of total polysaccharides (PS) and total proteins (PN), samples of 5 mL of granules were subjected to a cell lysis step with 10 mL of 1M NaOH solution and heated for 10 min in a water bath at boiling temperature. The PS measurement was conducted based on the method described by Dubois et al. (1956), whereas the PN quantification was carried out according to Bradford (1976).

4.3.5. Estrogens quantification

The estrogens quantification during Stage II was conducted in both the liquid phase (reactor influent, effluent and after feeding) and solid phase (biomass).

4.3.5.1. Liquid-phase quantification

For the liquid phase quantification, 200 mL liquid samples were concentrated by solid phase extraction (SPE) and then analyzed by high performance liquid chromatography (HPLC). Extraction was carried out using a solid phase cartridge Bond Elut C18 OH (Agilent Technologies 500 mg, 3 mL) and followed the methodology described by Bila et al. (2007). The extracts were resuspended in 1 mL of acetonitrile (Sigma Aldrich), filtered using sterile nylon membrane filters for syringe with 0.22 µm pore size, and transferred to 2 mL vials (Waters) for chromatography. The concentration of each estrogen (represented as E_i) were calculated using Equation (4.1).

$$[E_i] = \frac{R \; x \; V_{res}}{V_{ext}} \tag{4.1}$$

Where R is the concentration of resuspended sample (equipment response), V_{res} is the resuspended volume and V_{ext} is the extracted volume of the sample. The EDCs recovery was 90% for E2 and 71% for EE2.

The HPLC analyses were executed in a Waters Corporation® chromatograph equipped with a fluorescence detector and a Waters Novapak PAH C18 120 Å column (250 mm of length and 4.6 mm of internal diameter). The oven temperature was 30 ºC. The mobile phase was composed of acetonitrile (Tedia, Brazil) and ultrapure water

(obtained from Milli-Q apparatus, Millipore®) in a gradient elution method at a flow rate equal to 1 mL min^{-1} in the following proportions: 40% acetonitrile - 60% water during the first 6 min, 50% acetonitrile - 50% water during 3 min, 30% acetonitrile - 70% water for 4 min and 40% acetonitrile - 60% water for the last 2 min, giving a total run time of 15 min. The injection volume of each sample was 20 µL and the signal was received by a fluorescence detector with emission at 306 nm and excitation at 280 nm. The estrogens identification was carried out by comparing the retention time peaks of each individual compound from a stock solution of 10 mg L^{-1} containing E2 and EE2 in acetonitrile. For the estrogens quantification, a standard curve of E2 and EE2 in acetonitrile was prepared with concentrations of E2 (3.76 – 161 ppb) and EE2 (3.57 – 153 ppb) with 8 points of serial dilutions and linearity above 0.98 for both compounds. The limits of detection (LD) of the equipment were 0.99 and 0.51 µg L^{-1}, respectively, while the limits of quantification (LQ) were 3.76 and 3.57 µg L^{-1} for E2 and EE2. The data acquisition and processing of all data was performed in a Breeze 2 software (Waters Corporation).

The load of each estrogen fed to the SBR ($L_{Ei,IN}$ in µg) was calculated according to Equation (4.2), considering only the estrogens concentration in the aqueous phase, since there were no suspended solids in the synthetic medium used. The estrogen load in the effluent ($L_{Ei,OUT}$ in µg) was calculated by Equation (4.3).

$$L_{Ei,IN} = Q.C_{Ei,IN}.t \quad (4.2)$$

$$L_{Ei,OUT} = Q.C_{Ei,OUT}.t \quad (4.3)$$

Where Q is the influent flow rate of the reactor (L d^{-1}), $C_{Ei,IN}$ and $C_{Ei,OUT}$ represent the average concentrations (µg L^{-1}) of each estrogen in the influent and effluent, respectively, and t is the operating period (d).

4.3.5.2. Solid-phase quantification

The quantification of estrogens adsorbed on the granules was carried out following the methodology described by Ternes et al. (2002), with some modifications for aerobic granular sludge samples. First, a homogeneous sample of granules was taken from the reactor, macerated and centrifuged in a Quimis Q222T centrifuge at a speed of 1500 xg for 15 min. Subsequently, the supernatant was removed and the concentrated

samples were lyophilized in a Modulyod Freeze Dryer from Thermo Electron Corporation.

Once lyophilized, ultrasound extraction was performed, with 0.5 g of the lyophilized sample and 4 mL of methanol in an ultrasound (Thornton MS 200) for 10 minutes. This procedure was repeated two times for this solvent and then other two times with 3 mL of acetone. The mixture obtained in the tube was then centrifuged at 1500 xg for 5 min and the supernatant was collected. A 6 mL glass column containing silica gel (pore size 60 Å from Sigma-Aldrich) was used to clean the sample and eliminate interference from the matrix (GOMES, SCRIMSHAW, *et al.*, 2003). Silica activation was carried out by passing 5 mL of a 65:35 hexane/acetone solution through the silica gel column (v/v). Then, the sample from the ultrasound was transferred to the glass column and finally eluted with 5 mL of the same hexane/acetone solution. The sample collected in vials was dried by a nitrogen gas stream and then reconstituted with 1 mL of acetonitrile (Sigma Aldrich) for analysis in HPLC, following the methodology used for the quantification of estrogens in the liquid-phase.

Considering that the amount of estrogens fed to the reactor over time was constant, the amount of these compounds that adsorbs on and desorbs from the granules present within the AGS system during the cycle, at steady-state conditions, was also invariable. Thus, the amount of estrogens removed by adsorption on the granules ($L_{Ei,ADS}$ in μg) was calculated using Equation (4.4), as follows:

$$L_{Ei,ADS} = Q.C_{Ei,ADS}.TSS.t \quad (4.4)$$

Where, $C_{Ei,ADS}$ corresponds to the average concentration of the estrogens adsorbed on the biomass (μg g^{-1}), and TSS is the average concentration of the total suspended solids in the effluent (g L^{-1}).

By considering the estrogen load in the influent and effluent of the SBR, and the amount of estrogen removed by adsorption on the granular biomass, the amount of estrogens removed by biodegradation was determined according the Equation (4.5).

$$L_{Ei,ADS} = L_{Ei,IN} - L_{Ei,OUT} - L_{Ei,ADS} \quad (4.5)$$

4.3.6. YES assay

The estrogenic activity of the reactor effluent samples within Stage II was determined using a recombinant receptor gene assay in yeast cells, known as yeast estrogen screen (YES) test. The assay was performed according to the method of Routledge et al. (1996), with some modifications. A genetically modified yeast strain of *Saccharomyces cerevisae*, kept at room temperature (25 ºC), was replicated to be used in the tests. The methodology is based on the use of the enzyme β-galactosidase, which is synthesized by the yeast and metabolizes the chromogenic substrate CPRG (chlorophenol red-β-galactopyraneside), inducing color changes from yellow to red. The amount of this enzyme in the medium depends on the amount of estrogenic substance, so by spectrophotometric measurement of the absorbance it is possible to detect the amount of estrogen in the analyzed sample (ROUTLEDGE, SUMPTER, 1996).

First, 200 mL of the effluent were concentrated by solid phase extraction (SPE) following the methodology described by Bila et al. (2007), using solid phase cartridges Bond Elut C18 OH (Agilent Technologies 500 mg, 3 mL), and the extracts were reconstituted in 1 mL of ethanol (Sigma Aldrich). Sample extracts were serially diluted in absolute ethanol. For E2 standard curves, serial dilutions of an E2 stock solution (54.48 μ L^{-1}) prepared in ethanol (positive control) in the range between 2724 to 1.33 ng L^{-1}. Aliquots of 10 μL of each dilution were transferred to a 96-well optically flat microtitre plate and tested in duplicate. After total evaporation, 200 μL of yeast medium containing CPRG was added to the microtitre plate. The plates were sealed and placed on a shaker (IKA MS#) for 5 min and then incubated for 72 h at 30 ºC in a Quimis Q-316 M2 incubator. After the incubation period, the absorbance was read at 575 nm (for color) and at 620 nm (for turbidity) using a SPECTRAMAX M3 plate reader (Molecular Devices).

Dose–response curves for the E2 standard were obtained graphically by E2 concentration versus estrogenic response on corrected at 575 nm. The resulting sigmoidal curves were fitted to a symmetric logistic function using the Origin 6.0 software package (Microsoft, USA). The results were expressed as estradiol equivalent (EQ-E2). The EC_{50} mean value from the 17β-estradiol dose-response curve was of 24.8 ± 1.5 ng L^{-1} and the limits of detection (LD) and quantification (LQ) were 6 ng L^{-1} and 18 ng L^{-1}, respectively. The results of estradiol equivalent (EQ-E2) were obtained in ng L^{-1} by interpolation of the standard dose-response E2 curve with data from the samples.

During the YES assay, cytotoxicity may occur. Inhibition of yeast growth is visualized by the absence of turbidity at the bottom of the wells. In this case, as described by Frische et al., (2009), the absorbance control at 620 nm can be used as a tool to quantify the inhibition of yeast growth due to the toxicity of the samples, according to Equation (4.6).

$$Cytoxicity = 1 - \left(\frac{Abs_{620(sample)}}{Abs_{620(blanks)}}\right) \quad (4.6)$$

4.4. Results and Discussion

4.4.1. Continuous operation of AGS-SBR: performance on COD, nitrogen and phosphate removal

The performance of the AGS-SBR system was monitored according to the removal efficiencies of COD, nitrogen and phosphorus over 151 days of operation. Prior to the estrogens addition, the reactor was only fed with synthetic effluent (without the EDC solution) during 29 days (Stage I). This period served as experimental control for comparison with that when hormones were added to the reactor (Stage II).

The COD profiles over the entire operation of the reactor are shown in Figure 4.2a. The influent COD concentrations ranged between 350 and 500 mg L^{-1}, while the average value in the effluent was 34.7 mg L^{-1}. High COD removal efficiencies were achieved throughout the operation, with values above 90%, regardless of the presence or absence of EDCs in the reactor influent. From the COD mass balance assessment over the experimental cycles, it is possible to observe that about 87% of the influent organic matter was removed during the anaerobic feeding phase, and around 6% was later removed within the aerated period (Figure 4.3). The remaining (7%) was considered as non-biodegradable matter, possibly resulting from microbial degradation (ORHON, ARTAN, 1994) or consisting of minor components present in the influent stream. The anaerobic conversion of substrates into intracellular polymers is a desirable condition for achieving a stable AGS process. In fact, COD uptake in the presence of external electron acceptors (oxygen and/or oxidized nitrogen species – NOx^-) favours the proliferation of fast-growing organisms which may lead to loose and unstable granules (DE KREUK, VAN LOOSDRECHT, 2004).

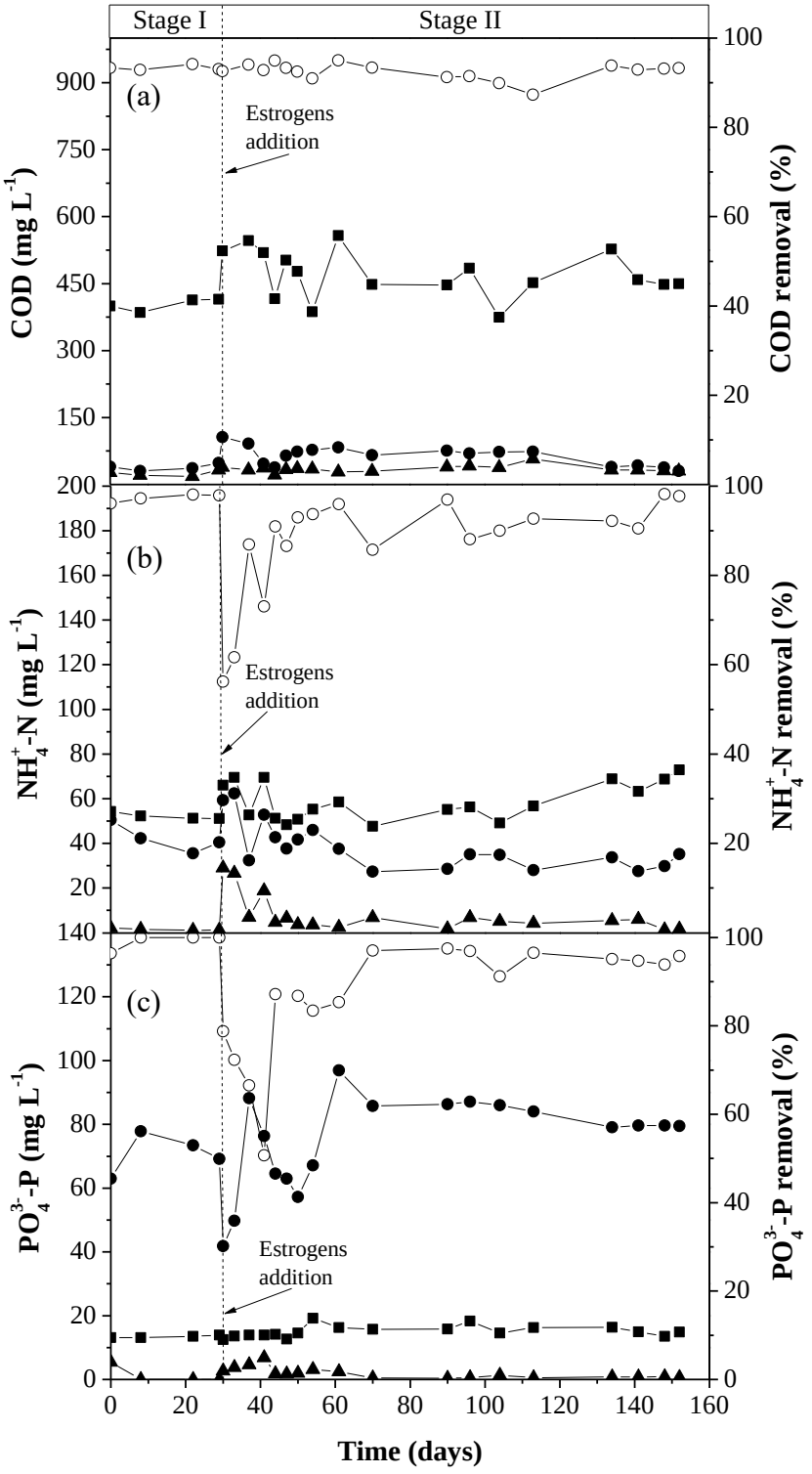


Figure 4.2 – Concentration profiles of a) Organic matter profiles expressed in terms of COD, b) ammonium and c) phosphorus in the influent (■), after the anaerobic feeding (●), in the effluent (▲) and removal efficiencies (o) during the entire operation.

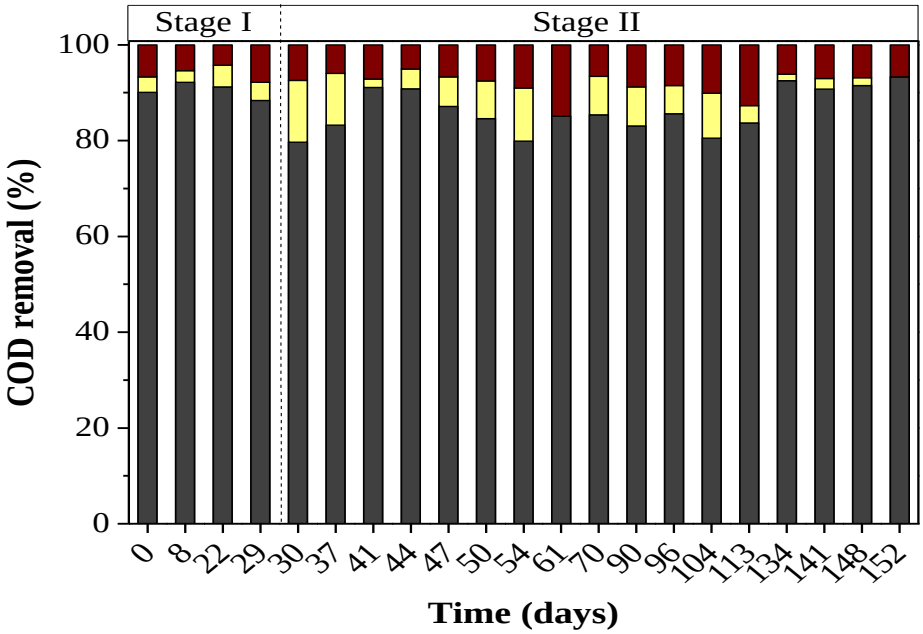


Figure 4.3 – COD contribution in each phase along the SBR cycle during the complete operation: ■ Anaerobic feeding phase, ■ Aerated phase and ■ remaining non biodegraded.

It should also be considered that COD removal may have partially been associated with denitrifying activity, given that the NO_x^- compounds remaining from the previous cycle (due incomplete total nitrogen removal, as will be discussed further) can be used as electron acceptor by denitrifiers to oxidize the influent organic matter (as acetate). Taking into account the NO_x^- present at the beginning of the cycle and that measured after the anaerobic step, the amount of NO_x^- reduced anaerobically was estimated. It corresponded, in average, to 4.6 mgN L^{-1}. Considering the theoretical COD/N ratio of 3.28 required for complete denitrification using acetate as electron donor and the hetetrophic sludge yield coefficient (Y) under anoxic conditions as 0.54 gVSS $gCOD^{-1}$ (CHOUBERT, MARQUOT, *et al.*, 2009), a COD of 32.8 mg L^{-1} was observed to be consumed for the complete reduction of nitrate via denitrification. This corresponds to less than 10% of the influent COD.

The ammonium nitrogen profiles are shown in Figure 4.2b. The influent concentrations varied between 48 and 68 mg L^{-1}, with an average value of 54 mg L^{-1}. After the feeding phase, the measured ammonium concentrations were lower than that of the influent due to the dilution of the latter with the remaining liquid from the previous cycle. During Stage I, period when there was no estrogen present in the reactor feeding,

ammonium removal was around 97%. However, upon addition of E2 and EE2 to the influent (Stage II), nitrification was severely affected, and ammonium removal reached only 58%. Consequently, high ammonium concentrations (23 – 35 mg L^{-1}) were detected in the effluent within the first 11 days of operation within Stage II. Inhibition of nitrifying organisms at concentrations higher than 10 µg L^{-1} of EE2 was reported by Khunjar et al., (2008). From days 41 to 47, ammonium removal remained very unstable. Nevertheless, despite the presence of estrogens in the influent, gradual recovery of ammonium oxidizing bacteria (AOB) activity after day 50 allowed to reach over 92% removal of this substrate. Concomitantly, residual ammonium in the effluent quickly decreased to values below 5 mg L^{-1}, condition sustained until the end of reactor operation. The maximum ammonium removal (98.2%) was achieved after 118 days of the reactor operation with EDCs (day 147). From the biomass production data, it was estimated that around 90% of the incoming nitrogen was removed via nitrification, while the remaining 10% was assimilated for biomass growth, the latter estimated by considering a nitrogen content in the biomass of 12% (METCALF & EDDY, 2003).

Soluble oxidized nitrogen products from nitrification (nitrite and nitrate) were also measured over the operation in order to better track the biological conversions and allow nitrogen removal to be assessed (Figure 4.4). Low nitrite levels were detected in the effluent (0.645 mgN L^{-1} in average), while nitrate corresponded to the main nitrification product in the SBR outlet stream, averaging 9.7 mgN L^{-1}. From the overall nitrogen balance conducted taking into account the average values of all soluble nitrogen species in the influent and effluent throughout the reactor operation, the total nitrogen (TN) removal was estimated to be around 71%. Of this total amount, 60% was removed via denitrification occurring simultaneously with nitrification, the later in the aerobic layers of the granular biomass and the first in the anoxic zones of the granules. Both overall TN removal and TN removal attributed only to denitrification are also shown in Figure 4.4. Simultaneous nitrification and denitrification (SND) process is commonplace in AGS-based processes due to the establishment of different redox zones within the granular structure (YAN, ZHANG, *et al.*, 2016).

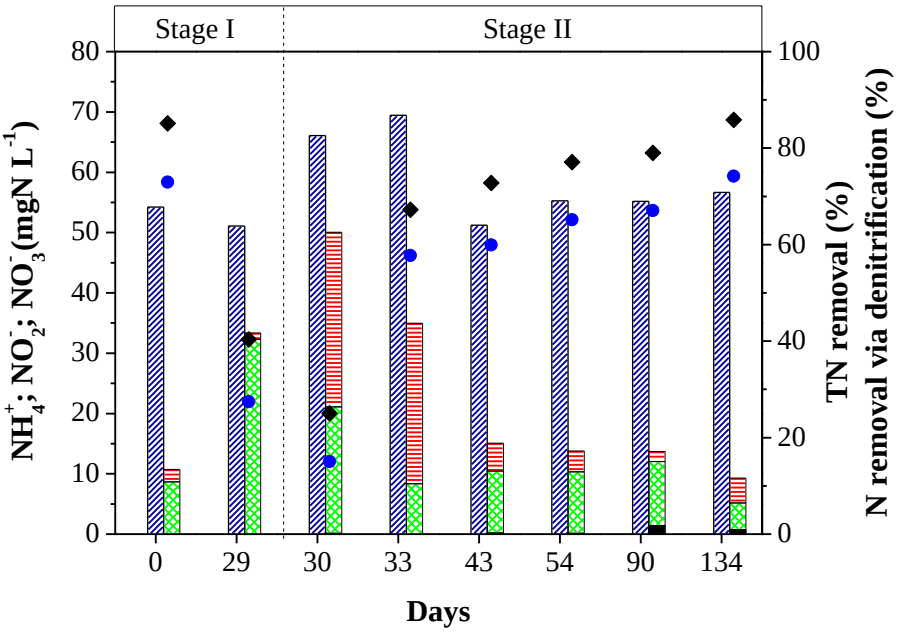


Figure 4.4 – Ammonium concentration in the influent (▬), ammonium (▬), nitrite (■), and nitrate (▬) concentrations in the effluent, total nitrogen removal (♦) and nitrogen removal via denitrification (●) over the operation.

For a better evaluation of the amount of nitrogen which was lost as gaseous nitrogen forms, Figure 4.5 shows the amount of ammonium effectively removed by nitrification (nitrified NH_4^+) and the corresponding NO_X^- (nitrate and nitrite) levels found in the effluent. The difference between these two represents the nitrogen which was denitrified, either via nitrite or nitrate in the anoxic regions of the granules.

The reason for not reaching higher TN removal levels is probably due to the high DO concentration kept inside the reactor, close to oxygen saturation levels. High DO concentrations reduce the anoxic region of the aerobic granules and consequently decrease the nitrifying activity (BASSIN, 2018). By affecting the distribution of aerobic and anoxic zones within the granular biomass, the DO level has a direct influence on TN removal efficiency. During the operation of an AGS-SBR, Yan et al. (2019) observed that when DO concentration increased from 0.7 to 1.2 mg L^{-1}, the TN removal decreased from 71 to 63%.

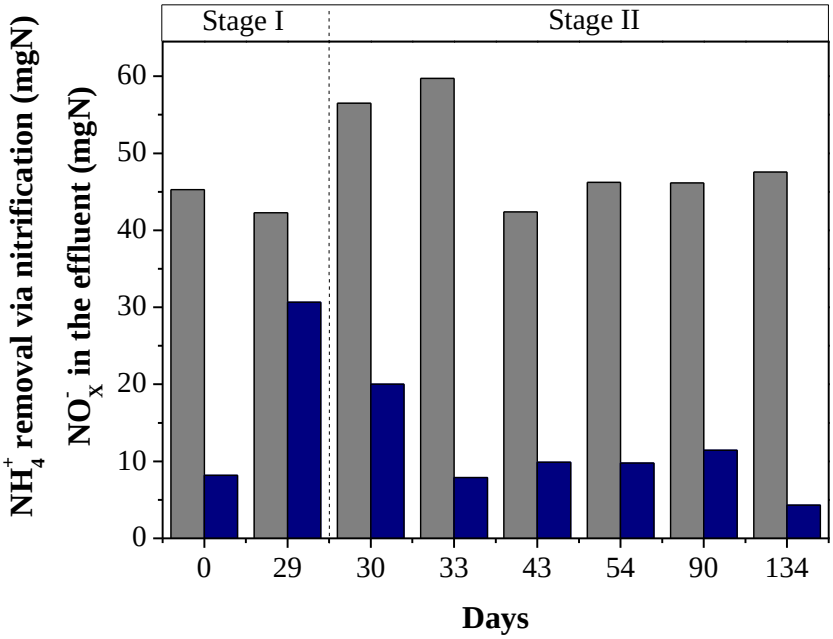


Figure 4.5 – NH_4^+ removed via nitrification (■) and NO_x^- (NO_2^- + NO_3^-) in the effluent (■) during the stages I and II.

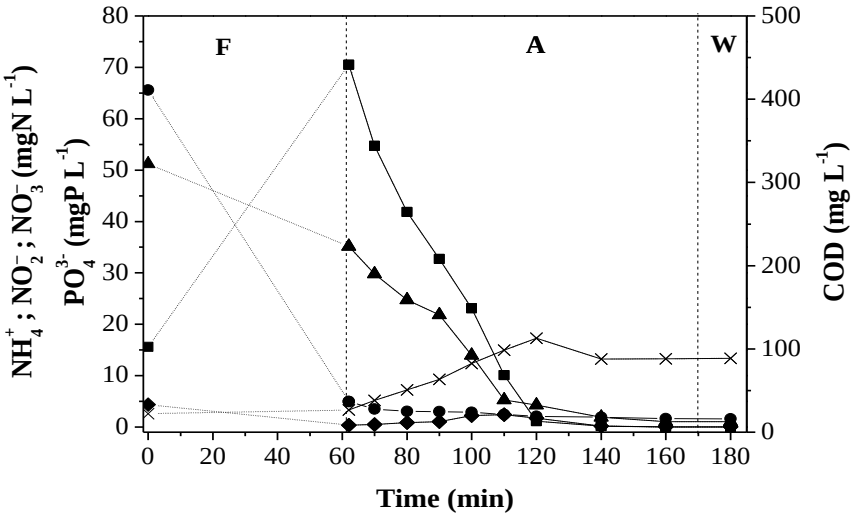
The phosphorus concentration profiles along the reactor operation are depicted in Figure 4.2c. The inlet concentrations varied between 12.4 and 19.1 mgP L^{-1}, with an average value of 15 mg L^{-1}. Anaerobic phosphate release and phosphate removal performance were very stable during Stage I, averaging 68.2 mgP L^{-1} and 100%, respectively. However, as observed for ammonium, the first 11 days of reactor operation within Stage II revealed an unstable EBPR process, during which large fluctuations in the amount of phosphate released anaerobically was observed (34 – 66 mgP L^{-1}). Nevertheless, 7 days upon EDCs addition, the phosphate release relative to the COD taken up during the anaerobic feeding period started to increase (from 0.08 to 0.15 mgP $mgCOD^{-1}$). PAOs assimilate the influent organic substrate and store it as intracellular polymers (i.e., polyhydroxyalkanoates) under anaerobic conditions. The energy for this conversion derives from the breakage of polyphosphate chains, causing phosphate to be released from the cells to the medium (VAN LOOSDRECHT, HOOIJMANS, *et al.*, 1997). Therefore, the increased P release per COD taken up suggests the recovery of PAO activity. Nevertheless, the residual phosphate detected in the effluent during this unstable period was still high and reached up to 6.5 mgP L^{-1}. This implies a substantially low phosphate removal (67% in average) at the beginning of the reactor operation with the presence of hormones in the inlet. Similar results were reported by Moreira et al. (2015),

who studied the performance of an AGS SBR system on the removal of the micropollutant fluoxetine. These authors observed drops in phosphorus removal upon supplementation of this compound to the influent stream at a concentration of 4 µM (1237 µg L^{-1}).

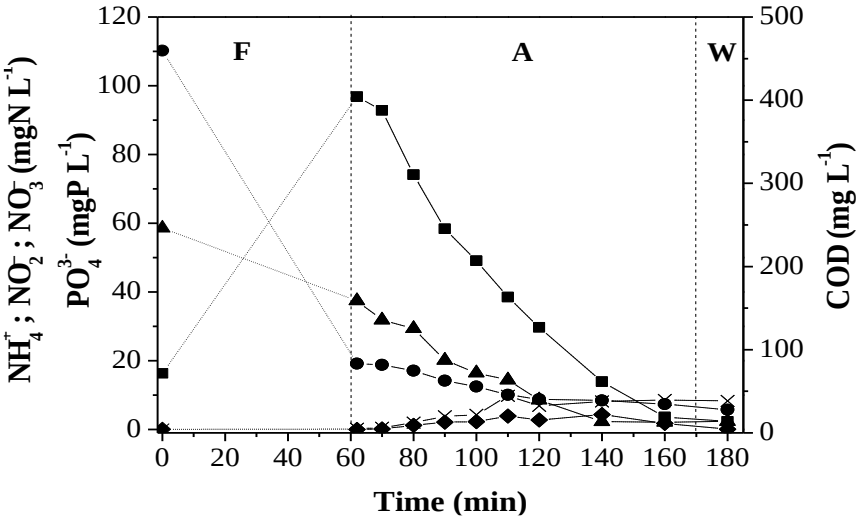
Despite the unstable biological phosphorus removal performance observed in the beginning of Stage II, a gradual adaptation of PAOs to the imposing conditions was observed from day 42, and phosphate concentrations in the effluent started to decrease until reaching a minimum of 0.46 mgP L^{-1}. Concomitantly, phosphate removal efficiency increased to 97% on day 70, and remained fairly stable until the end of the operation. The results indicate that the presence of estrogens seems to have an initial adverse effect on polyphosphate accumulating bacteria, but their long-term exposition to these compounds is accompanied by gradual adaptation and recovery, enabling a stable EBPR process to the re-established.

4.4.2. Substrate conversion rates as assessed by cycle tests

To follow the dynamics of the compounds over an operating cycle of the AGS-SBR to assess their conversion rates, cycle tests were carried out once steady-state conditions were achieved (invariant COD, N and P profiles) in both Stages I and II. The results are shown in Figure 4.6, while the specific conversion rates for phosphate and nitrogen species within Stages I and II are summarized in Table 4.2.



(a)



(b)

Figure 4.6 – Cycle tests performed on day 26 (Stage I) and 70 (Stage II) showing COD (●), ammonium (▲), phosphorus (■), nitrite (♦) and nitrate (×) profiles over the SBR cycle. The letters F, A and W refer to the different phases: feeding, aeration and effluent withdrawal, respectively. No sample was collected during the anaerobic feeding (non-mixed conditions), so the dashed line within this period represents only an indication of the trend (increasing or decreasing) of each compound.

For both experimental stages, the overall COD removal was similar, with slight differences observed for the organics conversions over the cycle. In stage I, 91% of the influent COD was taken up during the anaerobic feeding phase and the remaining 5% oxidized along the aerated phase. On the other hand, in Stage 2, the fraction of the incoming COD consumed during the feeding phase was 82% and the remaining (12%) was degraded during the aerated period. A small portion of the carbonaceous matter can be considered as non-biodegradable, as it remained constant throughout the aerated period, being therefore detected in the treated effluent (COD of around 16 mg L^{-1} in Stage I and 27 mg L^{-1} in Stage II). As discussed previously, this soluble inert COD fraction may result from microbial metabolism (ORHON, ARTAN, 1994) or be associated with the components used to prepare the synthetic wastewater.

Regarding the removal of ammonium by the nitrification process, a linear decrease of its concentrations was observed over the aerated phase for both stages. This substrate was rapidly oxidized within the first 60 min of the aerated period (120 min of the overall SBR cycle) and no further ammonium oxidation was observed after 140 min of the cycle, resulting in an effluent concentration of 1.1 mgN L^{-1} for Stage I and 2.3 mgN L^{-1} for Stage

II. The maximum biomass specific ammonium removal rate via nitrification (qNH_4) determined during the cycle test for Stages I and II was, respectively, 6.95 and 3.35 $mgNH_4^+$-N $gVSS^{-1}$ h^{-1}. The ammonium oxidation rate was therefore almost twice as high in the absence of EDCs, which implies that these compounds may have possibly caused an adverse effect on metabolic activity of nitrifying bacteria.

With respect to oxidized nitrogen species, an accumulation of nitrite was observed along the cycle in both stages, as a product of the ammonium oxidation, reaching a maximum value of 2.4 and 3.6 mgN L^{-1} for stages I and II, respectively. Subsequently, nitrite was oxidized to nitrate during the aerated phase, and the concentrations of this compound in the outlet stream were very low, around 0.02 mgN L^{-1} in Stage I and 0.1 mgN L^{-1} in Stage II. On the other hand, nitrate concentration gradually increased over the cycle as result of nitrite oxidation, and represented the main nitrification product in the effluent. The fact that nitrate formation was not observed at the same rate of the ammonium oxidation indicates that the later process, mediated by nitrifiers in the oxygen-containing outer layer of the granules, occurred simultaneously with nitrate reduction by denitrifying organisms in the inner (anoxic) part of the granular biomass. The specific denitrification rate was determined to 2.7 and 1.0 $mgNO_x^-$-N $gVSS^{-1}$ h^{-1} for the cycle test performed at steady-state conditions within Stages I and Stage II, respectively. This is similar to those values reported by Bassin et al. (2012b), who operated a similar AGS-SBR for simultaneous nitrogen and phosphate removal. However, their experiments were conducted at relatively low DO concentrations (1.8 mg L^{-1}).

The phosphate release during the anaerobic feeding phase was similar at the steady state conditions of both stages (68 and 75.2 mgP L^{-1} respectively), indicating a stable PAO activity regardless the presence or absence of EDCs in the influent. No phosphate was found in the effluent of Stage I, indicating full P removal, while in Stage II the effluent phosphate concentration remaining in the effluent amounted to 2.3 mgP L^{-1}. Specific phosphate uptake rates of 11.4 and 6.7 $mgPO_4^{3-}$-P $gVSS^{-1}$ h^{-1} were found for Stages I and II, respectively. As observed for specific ammonium oxidation, the specific activity of PAOs was almost twice as high in the absence of EDCs. These values are greater than that reported by Bassin et al. (2012c) who observed a maximum phosphate uptake rate of 3.9 and 5.9 $mgPO_4^{3-}$-P $gVSS^{-1}$ h^{-1} in AGS-SBRs accomplishing combined nitrogen and phosphate removal at 20 and 30 ºC, respectively. The P-uptake rates obtained in this study also were higher that the reported by Pronk et al. (2014), who

achieved a maximum specific phosphate uptake rate of 4.8 $mgPO_4^{3-}$-P $gVSS^{-1}$ h^{-1} working in an AGS-SBR subjected to a salinity level of 0.2 g Cl^- L^{-1}.

Table 4.2 – Specific conversion rates for phosphate and nitrogen compounds determined from the cycle tests performed for each experimental stage.

Stage	Specific NH_4^+-N uptake rate ($mgNH_4^+$-N/(g VSS.h))	Specific NO_x uptake rate ($mgNO_x^-$-N/(g VSS.h))	Specific PO_4^{3-}-P uptake rate ($mgPO_4^{3-}$-P/(g VSS.h))
I	6.95	2.7	11.4
II	3.35	1.0	6.7

4.4.3. Estrogens removal within Stage II

4.4.3.1. Assessing E2 and EE2 concentrations over time in both liquid and solid samples

Estrogens (E2 and EE2) concentrations in the effluent were regularly measured over 103 days after the beginning of Stage II, and the results are presented in Figure 4.7. The concentration of each estrogen in the influent wastewater was set at 20 µg L^{-1}, while their average concentrations in the effluent were observed to be 0.186 ± 0.03 and 1.4 ± 0.04 µg L^{-1} for E2 and EE2, respectively. This corresponds to a removal efficiency of 99% for E2 and 92.8% EE2, which is quite significant and higher than that reported by Balest et al. (2008), who found average removal efficiencies of 85% for E2 and 84% for EE2 using a sequencing batch aerobic granular biofilter reactor. It is interesting to note that EE2 proved to be more resistant to biodegradation than E2, despite the similarity between their molecular structures. This is due to the fact that the ethinyl group, located at position 17 of the EE2 estrogen, potentially blocks the formation of a ketone and sterically prevents access to the hydroxyl group in that same position, making its degradation more difficult than E2 (WEBER, S., LEUSCHNER, *et al.*, 2005).

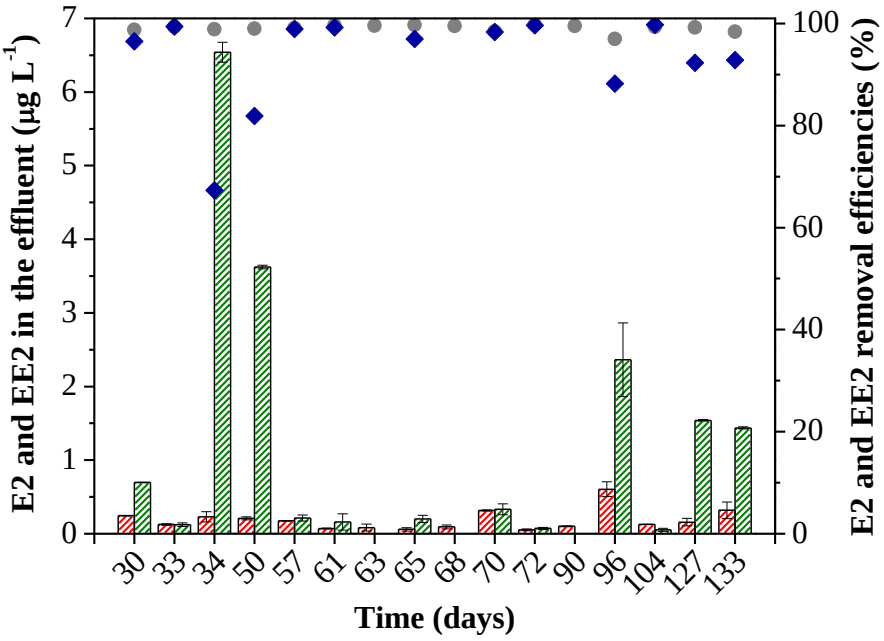
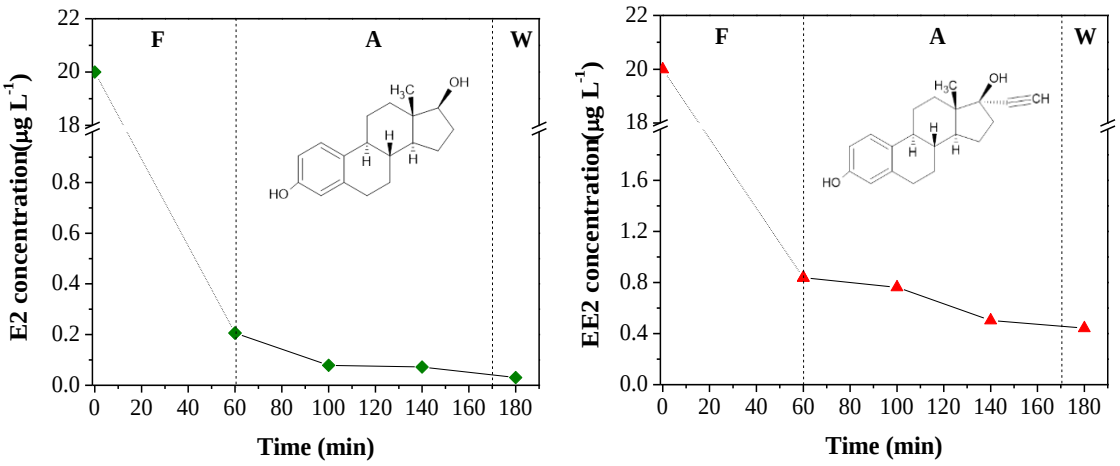


Figure 4.7 – Effluent concentrations of E2 (▨), EE2 (▨) and removal efficiencies of E2 (●) and EE2 (♦) over 103 days within Stage II. The influent concentration of E2 and EE2 was kept constant at 20 µg L^{-1}. The x-axis starts at 30 days because there was no EDCs addition to the influent within Stage I.

To better follow the micropollutants behaviour over the 180-min SBR cycle, a cycle test was performed and the results are plotted in Figure 4.8. The concentrations of both estrogens were sharply reduced during the anaerobic feeding phase to 0.21 µg L^{-1} (E2) and 0.84 µg L^{-1} (EE2). This represents a reduction of 98% (for E2) and 95% (for EE2) of their concentration in the influent stream. Further decrease of EDCs concentrations was noticed in aerated phase, but to a lesser extent compared to that observed anaerobically. The fast removal of estrogens from the liquid during the anaerobic phase is possibly associated with their physicochemical properties, especially their moderate hydrophobicity (log K_{ow} of 3.9 and 4.1 for E2 and EE2, respectively) and neutral charge. The EPS in microbial aggregates may also have an important function in the adsorption of organic and inorganic compounds. According to Sheng et al. (2010), several organic micropollutants can be adsorbed on the sludge due to the presence of a high number of carboxyl and hydroxyl groups in hydrophobic regions of the exopolymers, resulting in a high EPS adsorption capacity. Taking into account these properties of AGS, a rapid initial adsorption of these compounds into the outer layers of the granules may have occurred. Once adsorbed, they could have been degraded during

the aerated phase. As reported in the study by Hashimoto and Murakami (2009), all the estrogens evaluated (E2, EE2 and estrone (E1)) showed a high propensity to be adsorbed into activated sludge and were subsequently degraded under aerated condition.



(a) (b)

Figure 4.8 – Cycle test performed on day 147: (a) E2 (♦) and (b) EE2 (♦) concentration profiles over the SBR operating cycle. The letters F, A and W refer to the different phases: feeding, aeration and effluent withdrawal, respectively. No sample was collected during the feeding phase (non-mixed reactor), so the dashed line is just an indication of the trend of each estrogen during the anaerobic period.

As adsorption is reported to be an important mechanism of micropollutants removal in aerobic granular particles, the quantification of estrogens in the biomass was eventually carried out throughout the operation and the results are shown in Table 4.3. The amount of EDCs adsorbed onto the granules at the end of the SBR cycle was observed to vary over the operation. In the case of E2, an average of 0.033 µg g^{-1} was calculated to remain adsorbed on the biomass, while for EE2 it corresponded to 0.127 µg g^{-1}. Similar results were found by Luo et al. (2014) during the operation of MBBR systems subjected to a relatively low concentration of micropollutants in the feed (5 µg L^{-1}). The authors reported an absorbed amount of E2 and EE2 per gram of biomass of 0.025 - 0.05 µg g^{-1} and 0.1 - 0.5 µg g^{-1}, respectively. Considering the amount of biomass within the reactor in Stage II and the mass of EDCs fed to the reactor in each cycle, it can be estimated that only around 0.01% and 0.04% of E2 and EE2 were removed via adsorption.

Table 4.3 – Average concentrations of E2 and EE2 adsorbed on the aerobic granules at the end the cycle.

Day	E2 (μg g^{-1})	EE2 (μg g^{-1})
33	0.023 ± 0.98	0.048 ± 0.42
62	0.055 ± 0.02	0.068 ± 0.05
140	0.021 ± 0.07	0.267 ± 0.44

4.4.3.2. Insights into the potential mechanisms of EDCs removal in the AGS system

Although adsorption seems to play an important role in the removal of micropollutants, the concentrations of both E2 and EE2 adsorbed on the sludge at the end of the cycle were rather low, suggesting that this was not the main estrogens removal pathway. Seeking to better understand the main EDC removal mechanisms in the AGS-SBR system and their fate, a mass balance was carried out for these compounds during the operating period, considering both adsorption and biodegradation pathways. The results, listed in Table 4.4, are expressed as a percentage of their incoming concentrations, and show the main fates of both estrogens: biodegradation, adsorption onto sludge or discharge with the treated effluent (not removed).

Table 4.4 – Fate of the estrogens E2 and EE2 withdrawn with the treated effluent, adsorbed onto the biomass and biodegraded via bacterial metabolism.

Fate	E2 (%)	EE2 (%)
Discharged with the effluent (not removed)	0.93	7.1
Adsorbed onto the granules	0.01	0.04
Biodegraded	99.05	92.8

As it can be observed, not adsorption but biodegradation was the main estrogen removal mechanism in the AGS-SBR, contributing to the removal of 99% and 93% of E2 and EE2, respectively. The results obtained are in line with the reported by Margot et al. (2016), who reported natural estrogens (estrone and estriol) removal efficiencies by

biodegradation above 80% using aerobic granular sludge. They noticed that the removal of these compounds via adsorption was negligible (0.01% for E2 and 0.04% for EE2), but, as discussed previously, this mechanism may be important as the first step in the overall estrogens removal pathway, which is completed by biodegradation.

Evidences from literature studies show that depending on the availability of different electron acceptors such as O_2, NO_3^-, Fe^{3+} and SO_4^{2-}, microbial conversion rates associated with organic micropollutants removal may be favoured (CZAJKA, LONDRY, 2006). Moreover, there are several works suggesting a direct relationship between nitrification and biodegradation of micropollutants in biological systems (KHUNJAR, MACKINTOSH, *et al.*, 2011, MARGOT, LOCHMATTER, *et al.*, 2016, TORRESI, PLÓSZ, *et al.*, 2017, YI, HARPER, 2007). In fact, the biotransformation of steroidal compounds (e.g., EDCs) is mainly associated with co-metabolism pathways, since they are often present at low concentrations which are not enough to support biomass growth. Vader et al. (2000) studied the degradation of EE2 by a nitrifying activated sludge. They assigned the EE2 biotransformation to the AMO (ammonia monooxygenase) enzyme, produced by certain ammonium oxidizers during the first step of the nitrification process (i.e., oxidation of ammonium to nitrite), which is able to aerobically co-metabolise organic compounds. The study carried out by Yi and Harper (2007) also states the possibility of the AMO enzyme to act in the co-oxidation of steroid micropollutants within the nitrification process. Taking into account these previous reports, one potential EDCs biodegradation pathway occurs after the anaerobic feeding phase, when these compounds, already adsorbed on the granular biomass, are subsequently oxidized by nitrifiers during the aerated phase. The high ammonium oxidation rates observed in this study suggests a high activity of AOB and, therefore, of the AMO enzyme, corroborating this hypothesis.

Another possible EDCs biodegradation mechanism in the AGS-SBR system is via the denitrification process. As reported by Czajka and Londry (2006), anaerobic degradation from E2 to E1 in activated sludge systems occurred partially (56%) under nitrate reduction conditions with E1 accumulation, while EE2 biodegradation was not observed. Zeng et al. (2009) also reported that EE2 was almost completely biodegraded (97%) in an activated sludge system under anoxic conditions, with nitrate as an electron acceptor. In our study, denitrification occurs in the anoxic zones of the granules during the aerated period but also to a lesser extent during the anaerobic feeding phase. In the first case, NO_x^- generated by nitrification is mainly reduced by PAOs and glycogen-

accumulating organisms (GAOs), which use the intracellularly stored polyhydroxyalkanoates (PHA) as electron donor. Denitrification by other ordinary heterotrophs during aeration is less likely to occur, as external COD was almost completely consumed in the previous anaerobic phase by PHA-storing organisms. On the other hand, in the second case, the NO_x^- remaining from the previous cycle (due incomplete TN removal) is used as electron acceptor by ordinary denitrifiers to oxidize the influent organic matter (as acetate). The highest denitrifying activity occurs during aeration, where the amount of NO_x^- reduced reached up to 39.2 mgN L^{-1} as compared to only 4.6 mgN L^{-1} reduced anaerobically. However, it should be noted that denitrification within the aerated period is mainly mediated by either denitrifying PAOs and GAOs. Although the potential role of these organisms in the biodegradation of EDCs can be speculated as both microbial functional groups are important for organic matter, phosphate and nitrogen conversions occurring within the AGS-based system, there is no study reporting this conversion. What is actually well known is that these organisms prefer readily biodegradable substrates, such as volatile fatty acids (acetate, propionate, and others), which can be converted into PHA. Under these circumstances, their relevance for EDCs removal is unlikely. Taking into account the observations, it can be hypothesized that, in addition to the rapid adsorption of EDCs onto aerobic granules, an initial biodegradation of estrogens by denitrifiers may also have occurred during the anaerobic feeding phase, being completed during aeration.

Besides the potential role of nitrifying and denitrifying organisms on EDCs biodegradation, aerobic heterotrophic microorganisms may also have contributed to this conversion during the aerated period of the SBR. During this period, limited organic carbon was available, as most of the influent COD has been removed anaerobically, by either PAOs or GAOs. On average, only 12.8% of the incoming COD was still present when the aerated period started (Figure 4.3). Therefore, the limiting amount of external COD may have stimulated ordinary aerobic heterotrophs to use the micropollutants for their metabolism. McAdam et al. (2010) found that 51% of E1, E2, E3 and EE2 were degraded in an activated sludge reactor in the absence of nitrogen sources, and therefore, with heterotrophs as dominant organisms. Similar removal percentages for the same EDCs (E1, E2, E3 and EE2) were observed by Bagnall et al. (2012), who investigated their biodegradation in an activated sludge system in the absence of ammonium. Vilela et al. (2020) also reported that only heterotrophic bacterial strains have grown on EE2 as

the sole carbon source, with these organisms being possible candidates for the assembly of an estrogen-degrading consortia.

Considering the results of this study and data presented in previous works, a schematic representation of the possible EDCs degradation mechanisms by several microbial groups is displayed in Figure 4.9. It is assumed that both nitrifying and denitrifying bacteria are important for E2 and EE2 biodegradation, although the role of ordinary aerobic heterotrophs in this conversion cannot be excluded. Further experiments should be conducted for better assessment of EDCs degradation pathways in AGS systems, in which a complex bacterial consortium is harboured.

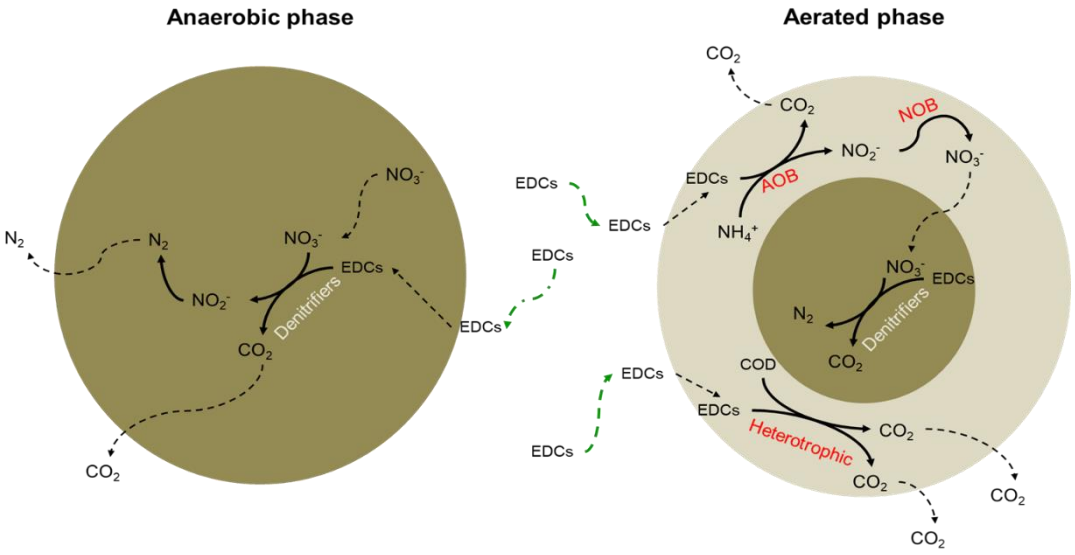


Figure 4.9 – Schematic representation of the proposed EDCs biodegradation pathways in the aerobic granules: aerobic zone (■), anoxic zone (■), conversion (arrow), diffusion (dot arrow), adsorption (green dot arrow).

4.4.4. Estrogenic activity

The estrogenic activity of the samples collected regularly at the outlet stream of the AGS-SBR system was assessed by the YES assay. E2 estrogen was used as a standard for the estrogenic activity test, and therefore consisted of the positive control for comparison with the estrogenic activity of the analysed samples. The E2 standard curve (Figure 4.10) presented a sigmoidal shape, adjusted by a nonlinear regression method (ROUTLEDGE, SUMPTER, 1996). It is important to note that cytotoxicity (yeast cells inhibition by the action of estrogen) was not shown in any of the samples analysed, which

indicates that the obtained estrogenic potential is not underestimated due to yeast inactivation or death.

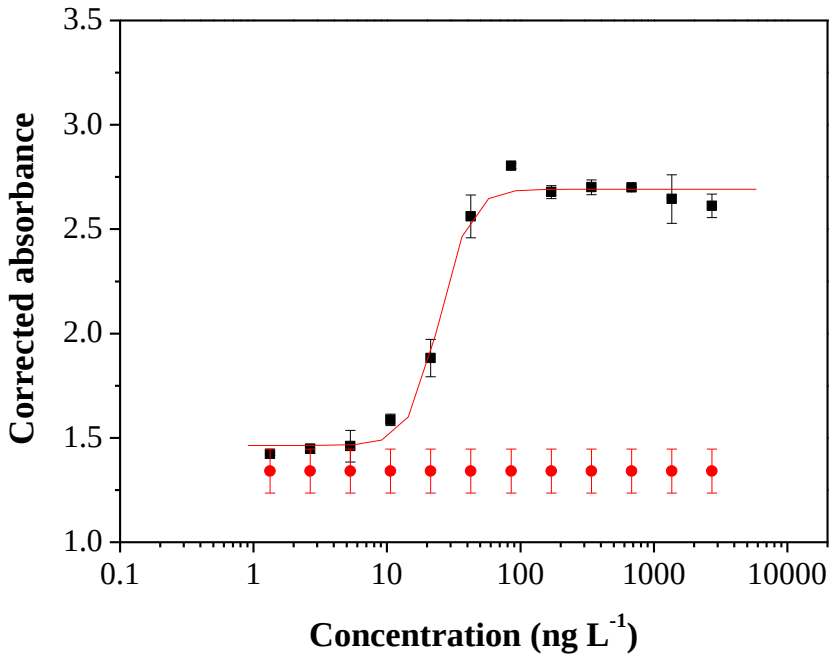


Figure 4.10 – Dose-response curve of positive control E2 (■) and negative control (●) in the YES assay.

Estrogenic activity was expressed as equivalents of E2 (EQ-E2). The EQ-E2 compares the estrogenic potential of the analysed samples with the estrogenic potential of E2. The results, shown in Table 4.5, indicate that most of the samples collected downstream of the biological process had low residual concentrations of EQ-E2 (below 0.055 µg L^{-1}), with the exception of that collected on day 70 (0.122 µg L^{-1}).

Table 4.5 – Residual E2 and EE2 concentrations and EQ-E2 values of the concentrated samples from the AGS-SBR process over the reactor operation.

Day	E2 (µg L^{-1})	EE2 (µg L^{-1})	EQ-E2 (µg L^{-1})
68	0.094	<LD[a]	0.011
70	0.314	0.33	0.122
93	0.101	<LD[a]	0.014
104	0.129	0.052	0.052

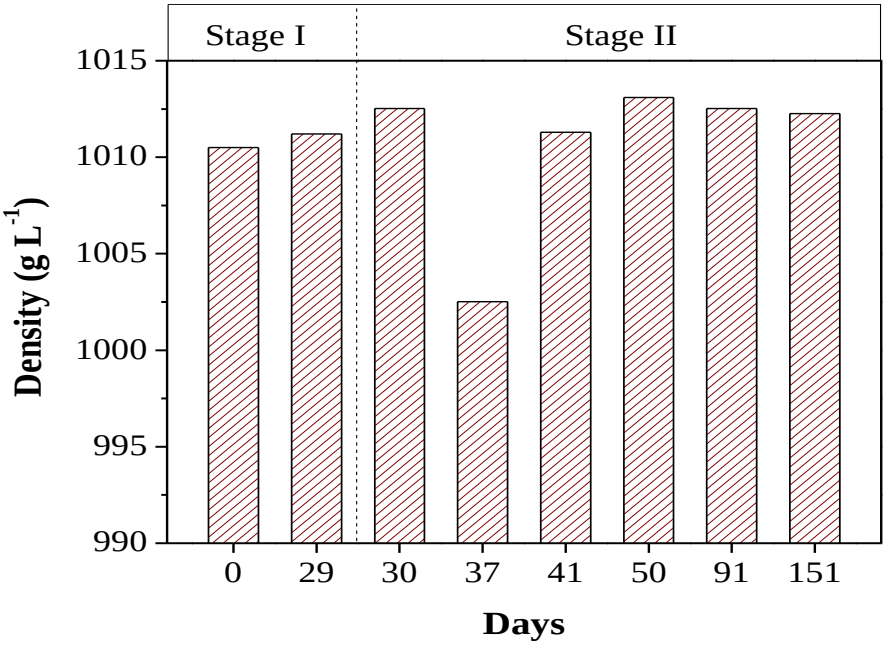
[a] Below the limit of detection

It is also interesting to observe that the residual concentrations of E2 and EE2 samples were higher than EQ-E2 values. This behaviour can be caused by the presence of any enzyme or other compound originated during the biological process as a result of microbial metabolism, which is complexed with estrogens and, therefore, does not allow estrogenic activity to manifest completely in the most concentrated well of the microlitre plate used in the test.

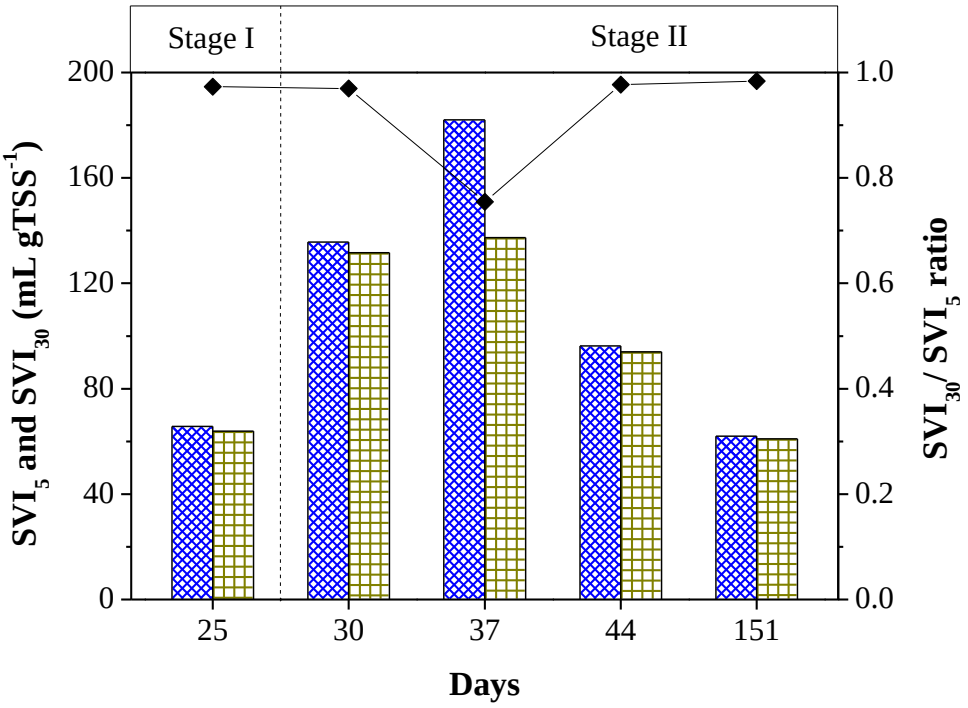
Although the residual concentrations of both hormones are rather low, they can still be harmful to the endocrine system of aquatic organisms (JAROŠOVÁ, BLÁHA, *et al.*, 2014). As the results obtained by HPLC/FLU showed a high biodegradation of both compounds (> 90%), it is possible that the estrogenic potential detected in the YES assay is associated with another type of intermediate estrogenic compounds formed from the biodegradation of E2 and EE2.

4.4.5. Physical properties of the granular biomass

The density of the granular biomass over time is displayed in Figure 4.11a. Within Stage I, the granules density was around 1011 g L^{-1}, considered normal for stable granules (WINKLER, KLEEREBEZEM, *et al.*, 2013), and similar values were maintained even at the beginning of Stage II. However, EDC addition to the reactor influent caused an impact on the physical characteristics of the granules during the first 11 days under these conditions, with the density dropping to 1002 g L^{-1}. At the end of Stage I, the SVI_5 and SVI_{30} were found to be 66 and 64 mL $gTSS^{-1}$, giving a SVI_{30} SVI_5^{-1} ratio of 0.97, which indicates good biomass settling properties (Figure 4.11b). After EDC addition, SVI_5 and SVI_{30} values increased to 136 and 132 mL $gTSS^{-1}$ respectively, but the SVI_{30} SVI_5^{-1} ratio (0.96) still indicated good settling conditions. However, 7 days after EDC addition, the density sharply dropped to 1002 g L^{-1} and, concomitantly, SVI_5 and SVI_{30} increased to 182 and 137 mL $gTSS^{-1}$ respectively, leading to a SVI_{30} SVI_5^{-1} ratio of 0.75, a value not compatible with stable aerobic granules. As regards to the particles size, the larger fraction of the granules (41%) during Stage I exhibited a mean diameter varying between 1 and 2 mm, a value reported by different authors as ideal for mature aerobic granules (DE KREUK, DE BRUIN, 2004, BASSIN, 2018). Similar diameter values were observed for the granular biomass during Stage II, regardless of the presence of EDCs in the incoming wastewater.



(a)



(b)

Figure 4.11 – a) Average density of the granules and b) sludge volumetric index (SVI) for 5 min (▦) and 30 min of sedimentation (▦); and SVI_{30}/SVI_5 ratio (♦) over the Stages I and II of the SBR operation.

VSS and TSS concentrations in the reactor remained relatively constant during operation within Stage I and in the beginning of Stage II. However, a few days after estrogens addition, TSS and VSS concentrations increased from 9 to 14 g L^{-1} and 6.8 to 10.6 g L^{-1}, respectively (Figure 4.12a). VSS TSS^{-1} ratio was found to be 0.76 in Stage I, decreasing to 0.62 upon EDCs addition. Nevertheless, this ratio increased again to 0.74 in the long-term operation within Stage II.

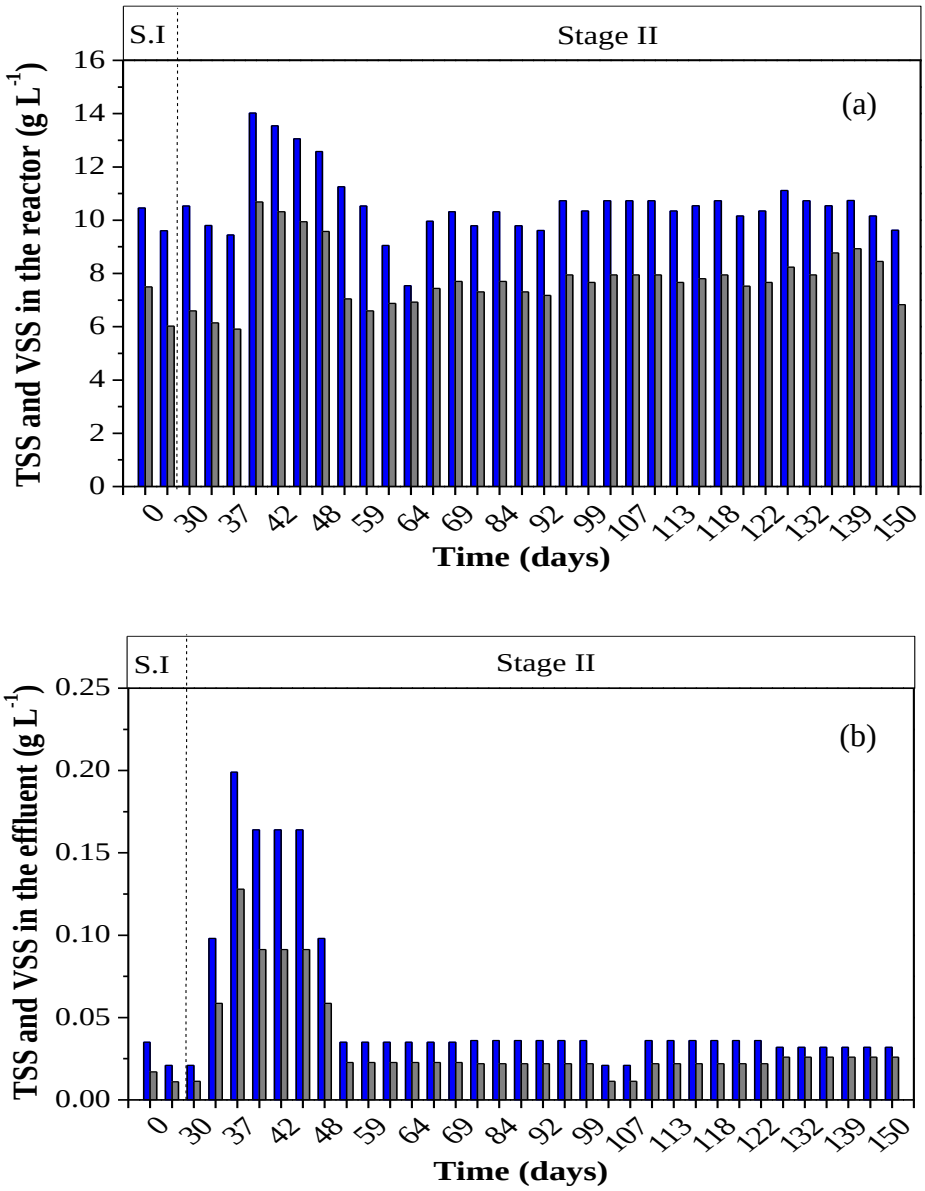


Figure 4.12 – Total suspended solids (■) and volatile suspended solids (■): a) inside the reactor and b) in the effluent during the entire operation. S.I indicates Stage I.

The decrease in the density of the granules at the beginning of Stage II was associated to the presence of the external agent (EDCs) that caused a disintegration of the granules surface and affected their stability. This effect was reflected in the effluent concentration of total (TSS) and volatile (VSS) solids, which normally were, respectively, in the range of 0.02 and 0.01 g L^{-1} (Stage I), but increased to 0.19 and 0.13 g L^{-1} after the estrogens addition (Figure 4.12b). Taking into account the entire operation of the reactor, the average ratio VSS TSS^{-1} in the effluent was 0.64.

Another important characteristic observed within the first 15 days of Stage II was the two-fold increase in turbidity of the effluent, reaching 10.2 NTU, while in Stage I it amounted to 5.08 NTU. A possible explanation for this effect was the greater production of extracellular polymeric substances (EPS) by aerobic granules. Among the extracellular polymers, proteins (PN) and polysaccharides (PS) are the most important ones as they contribute for microbial aggregation and therefore granules development (SHENG, YU, *et al.*, 2010). Quantification of PN and PS was therefore performed before and after adding estrogens to the reactor and the results are displayed in Figure 4.13.

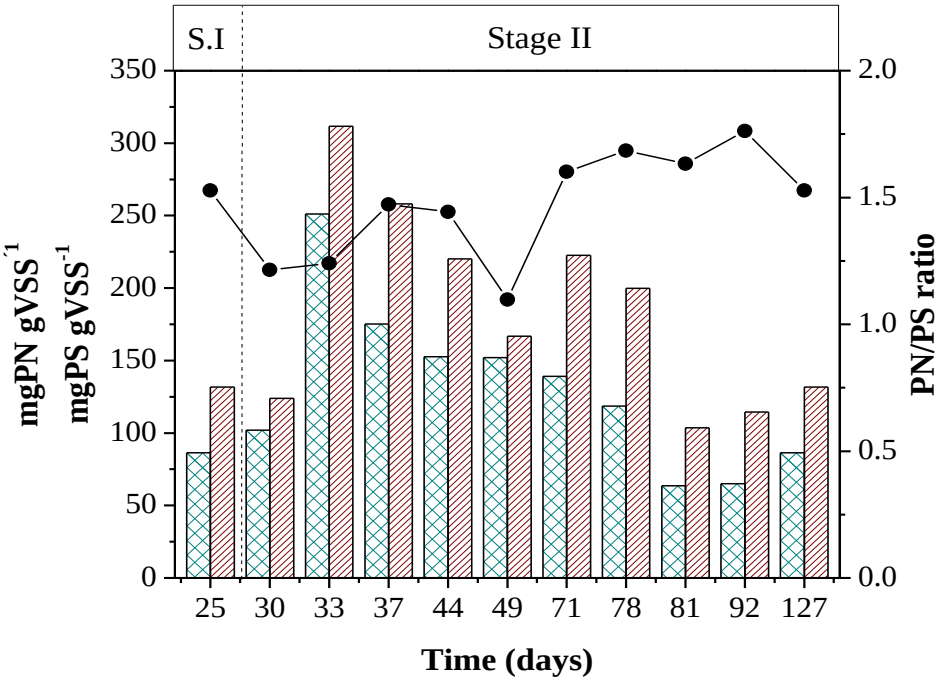


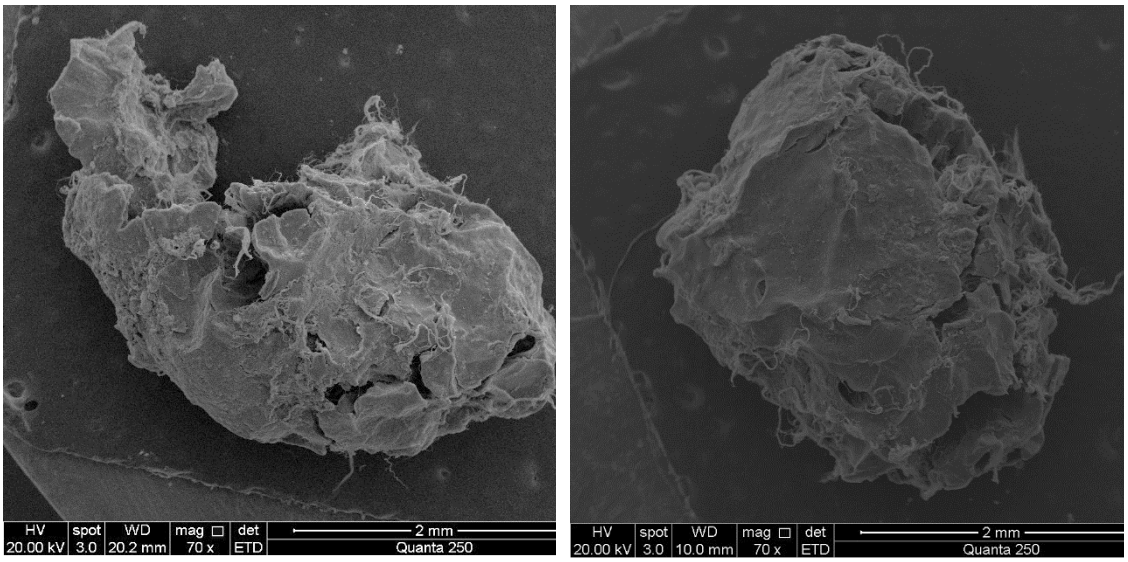
Figure 4.13 – Proteins (▨) and polysaccharides (▩) within the granular biomass during the SBR operation. S.I represents the Stage I.

Taking into account the entire operating period of the SBR, the average PN and PS concentrations within the biomass were 185.2 mgPN $gVSS^{-1}$ and 130.5 mgPS $gVSS^{-1}$ respectively. The results corroborate those reported by McSwain et al. (2005) and Zhu et al. (2015), whose research have also shown that proteins were the predominant substances present in the EPS of aerobic granular sludge. It is also possible to observe that there was an increase in the PN and PS levels after the addition of estrogens to the reactor inlet, from 131 mgPN $gVSS^{-1}$ and 86 mgPS $gVSS^{-1}$ on day 25 (Stage I) to 311.5 mgPN $gVSS^{-1}$ and 251.2 mgPS $gVSS^{-1}$ on day 33 (Stage II). This increase may be associated to a protection mechanism shown by microorganisms when they are exposed to the estrogens, which may have caused toxicity to them. In fact, the slight disintegration of the granular particles when subjected to EDCs may have forced the organisms to release more EPS to maintain their structural integrity (AVELLA, ESSENDOUBI, *et al.*, 2010). The higher production of EPS by aerobic granules upon estrogens supplementation to the reactor influent coincided with the increase of solids in the reactor (Figure 4.12a), so that these two variables were possibly associated with each other.

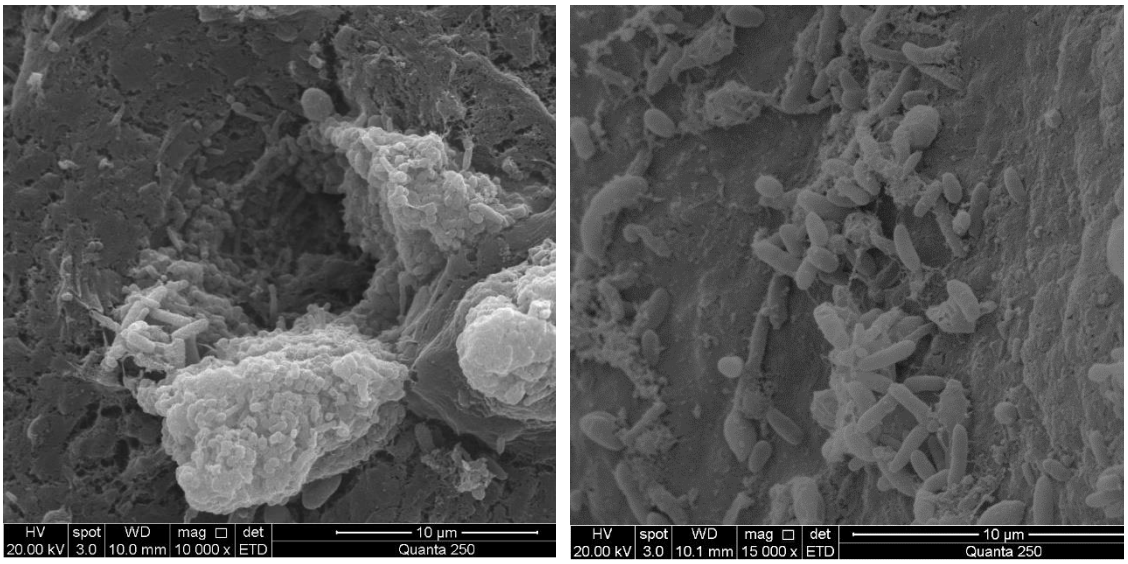
Despite the disturbances caused by the presence of EDCs at the initial part of Stage II, after approximately 11 days of operation within this experimental condition, the density of the granules began to return to the original values and increased to 1011 g L^{-1} (Figure 4.11a). The average density within this stage was found to be 1012 g L^{-1}. At the same time, the content of volatile and total solids in the effluent decreased, returning to typical values of 0.03 gTSS L^{-1} and 0.02 gVSS L^{-1}, respectively (Figure 4.12b). The SVI_5 and SVI_{30} values gradually decreased over Stage II, reaching 62 and 61 mL $gTSS^{-1}$ respectively, on day 151. This gives a SVI_{30} SVI_5^{-1} ratio close to 1, indicating that the good settling properties of the sludge was re-established (Figure 4.11b). The effluent turbidity also diminished and the PN and PS contents within the granules started to decrease again (Figure 4.13). PN PS^{-1} ratios varied from 1.09 – 1.76 and averaged 1.55. These changes were accompanied by the increase in the removal of COD and nutrients (Figure 4.2), allowing the system to be kept steadily stable even in the presence of estrogens until the end of reactor operation.

Scanning electron microscopy (SEM) images were obtained from granules samples after the start of the SBR operation under the influence of EDCs. In Figure 4.14a, it is possible to observe that there was indeed a disintegration in the structure of the granules when in contact with the estrogen compounds. Cracks and small imperfections were observed mainly in the outer layers of the granular sludge particles, which

negatively affected their physical properties and stability. The presence of some filamentous microorganisms around the granules can also be observed, which may have caused a decrease in the biomass density, as previously discussed. After 14 days of operation within Stage II, when the system stabilized and the physical properties of the granular biomass were re-established, SEM images revealed the predominance of dense granules with well-defined spherical appearance, indicating the recovery of their original structure and adaptation to the EDCs present in the wastewater (Figure 4.14b). The images also allow to observe the internal structure of the granules, showing clusters of microorganisms of various types and morphologies, mainly round (cocci) (Figure 4.14c) and rod-shaped (bacilli) (Figure 4.14d) organisms.



(a) (b)



(c) (d)

Figure 4.14 – SEM images of the mature aerobic granules samples obtained from the SBR: (a) 3 days after estrogens addition to reactor feeding; b) 14 days of operation within Stage II; c) inner part of the mature granule showing microbial cells aggregated into EPS matrix; and d) different bacterial cells located on the inner slices of the granules.

4.5. Conclusions

The results of this study have shown that the AGS-SBR was effective in the simultaneous removal of organic matter, nutrients and EDCs (E2 and EE2, both at 20 µg L^{-1}). Overall COD, ammonium, total nitrogen, and phosphate removal were found to be 93%, 87%, 71%, 87%, respectively, while E2 and EE2 removal percentages reached 99% and 93%, respectively. Although adsorption seems to be an important step for EDCs removal, biodegradation was the main removal pathway of these compounds. In fact, most of E2 (99%) and EE2 (93%) removal was achieved by microbial degradation routes. The results also shown that the AGS reactor was able to substantially minimize the estrogenic potential of steroid compounds, as evidenced by YES bioassays. Further studies should exploit the potential of AGS-based systems in the removal of micropollutants from real wastewaters, a topic of growing concern for environmental protection agencies and general society worldwide.

5. Tube-in-Tube Membrane Reactor for Heterogeneous TiO_2 Photocatalysis with Radial Addition of H_2O_2

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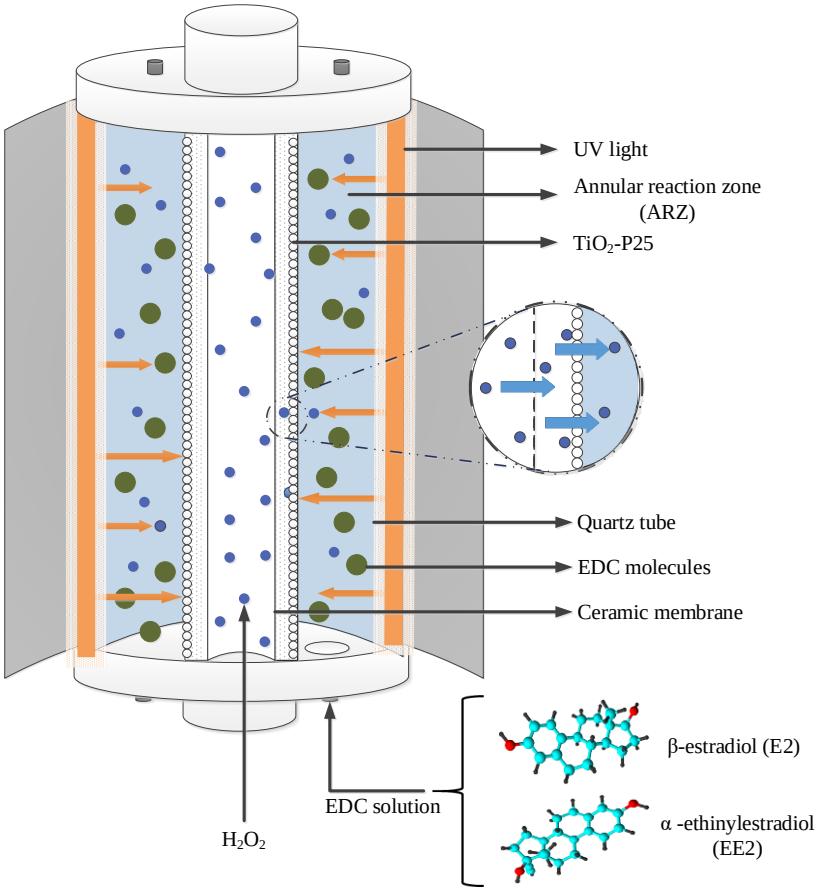
5.1. Graphical abstract



5.2. Introduction

Endocrine Disrupting Chemicals (EDCs) are a group of substances that can disturb the endocrine system in animals or humans, leading to adverse effects in the hormonal control or even cancer (ESPLUGAS, BILA, *et al.*, 2007, ZHANG, Y., ZHOU, 2008, BECKER, RODRIGUEZ, *et al.*, 2017). Natural and synthetic estrogens, such as 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are EDCs that have been detected in several water compartments (WANG, G., MA, *et al.*, 2012, ZHANG, C., LI, *et al.*, 2016, WOODING, ROHWER, *et al.*, 2017, VILELA, BASSIN, *et al.*, 2018). Urban wastewater treatment plants (WWTPs) are one of the main anthropogenic sources of EDCs and other contaminants of emerging concern (CECs) due to inefficiency of conventional activated sludge systems to completely remove them (HARRISON, RAYNE, *et al.*, 2006, TIJANI, FATOBA, *et al.*, 2013, ZWART, JONKER, *et al.*, 2020).

Advanced oxidation processes (AOPs), such as UVC/H_2O_2, anodic oxidation, Fenton, photo-Fenton, TiO_2 photocatalysis, ozone driven processes and others, have been tested for EDCs removal from different water matrices (DRURY, SNYDER, *et al.*, 2006, ZHAO, HU, *et al.*, 2008, DE LA CRUZ, GIMÉNEZ, *et al.*, 2012). However, these AOPs normally use expensive chemicals, catalysts and photocatalysts, some of them harmful, which need to be reclaimed. Namely, one of the main problems associated with the use of Fenton (Fe^{2+}/H_2O_2) or TiO_2 based AOPs is the use of massive amounts of dispersed iron and nanoparticulate TiO_2 that are hard to recover after the wastewater treatment (STASINAKIS, 2008). Also, TiO_2 nanoparticles are potentially harmful for humans (MA, ZHANG, *et al.*, 2015).

Membrane technology provides excellent separation efficiency and high treated water quality; nevertheless it still also has some drawbacks: membrane fouling, limited flux, lack of ability to degrade pollutants (BENOTTI, STANFORD, *et al.*, 2009, OJAJUNI, SAROJ, *et al.*, 2015, SZYMAŃSKI, MORAWSKI, *et al.*, 2017). To overcome such limitations, membrane functionalization with nanoparticles are a good approach (nano-engineered membrane-NEM). Ceramic NEM allows only the permeation of small organic molecules and water through the membrane pores and concurrently its photocatalytic activity results in the degradation of the larger organic pollutants present in water (BENOTTI, STANFORD, *et al.*, 2009, SARASIDIS, PLAKAS, *et al.*, 2014, GANIYU, HULLEBUSCH, *et al.*, 2015, ESPÍNDOLA, CRISTÓVÃO, *et al.*, 2019). Furthermore, the immobilization of the photocatalyst in an inert support (e.g. membrane) is a very interesting approach, since avoids a post-filtration step to remove the nanomaterials dispersed in the water, if slurry photocatalyst suspensions are used.

Literature also reports the use of membranes as ozone distributor, generating minute ozone bubbles in water, boosting the mass transfer rate from gas to liquid phase and the organic pollutants oxidation and, at the same time reducing the amount of oxidant necessary for the reaction (MOZIA, 2010, SARASIDIS, PLAKAS, *et al.*, 2014, ESPÍNDOLA, SZYMAŃSKI, *et al.*, 2019). Vilar et al. (2020) proposed a tube-in-tube membrane reactor for photochemical UVC/H_2O_2 processes where H_2O_2 is injected radially, resulting in a more homogeneous distribution of the chemical across the entire reactor length.

This work proposes the use of a disruptive tube-in-tube membrane reactor for continuous "titration" of small amounts of H_2O_2 to the active catalyst sites immobilized in the membrane shell-side (heterogeneous H_2O_2/TiO_2 photocatalysis), improving its

contact with the catalytic sites and pollutants to be oxidized, also avoiding catalyst deactivation. This system allows the integration of membrane technology with TiO_2 photocatalysis and UVC/H_2O_2 photochemical systems within a single unit. The oxidation of 17β-estradiol and 17α-ethinylestradiol in the novel tube-in-tube membrane reactor was determined as a function of the amount of TiO_2-P25 deposited on the outer surface of the ultrafiltration membrane, H_2O_2 dosing rate, H_2O_2 addition mode (radial permeation through the porous inner membrane or injection upstream from the reactor inlet), light source (UVC or UVA lamps) and aqueous solution matrix.

5.3. Materials and Methods

5.3.1. Reagents

17β-estradiol (E2, MW = 272.38 g mol^{-1}, 98% w/w purity, CAS# 50-28-2) and 17α-ethinylestradiol (EE2, MW = 296.403 g mol^{-1}, 98% w/w purity, CAS# 57-63-6) were supplied by Sigma-Aldrich. E2/EE2 stock solution (20 mg L^{-1}) was prepared in methanol. Titanium dioxide Aeroxide® P25 powder (TiO_2-P25) (Evonik, >99.5% w/w purity, average particle size = 21 nm, BET specific surface area = 50 ± 15 m^2 g^{-1}, density = 3.9 g cm^{-3}, crystalline phases: 20% wt. rutile and 80% wt. anatase) was employed as catalyst. The surfactant Triton™ X-100 (Sigma-Aldrich) was added to the TiO_2-P25 nanoparticles suspension. H_2O_2 (30% v/v) was supplied by Labkem and used as oxidant. Sodium sulfite (Na_2SO_3, p.a) supplied by Merck, was added to E2/EE2 aqueous solution samples to stop the degradation process in a Na_2SO_3-H_2O_2 molar ratio 1:1 (LIU, W., ANDREWS, *et al.*, 2003). Ammonium metavanadate (Merck, p.a) was used in the H_2O_2 analytical method. For E2 and EE2 determination by HPLC-DAD, acetonitrile (Merck, p.a) and ultrapure water were used as mobile phases. The synthetic E2/EE2 solutions and the mobile phases for HPLC system were prepared with pure (Panice® reverse osmosis system) and ultrapure water (Millipore® Direct-Q system), respectively.

An effluent sample collected downstream from the secondary settling tank of a municipal WWTP located in Northern Portugal was characterized (Table 5.1) and kept refrigerated until use. Pure water (PW) and real (UWW) matrices, both spiked with 100 μg L^{-1} of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), were used as inlet streams of the tube-in-tube membrane reactor. The pH of UWW was adjusted to 5.0, 6.0 or 7.0, with sulfuric acid (Pronalab, purity 96%, 1.84 g cm^{-3}) whenever required.

Table 5.1 – Physicochemical characteristics of the secondary urban wastewater.

Parameters (units)	
Color	Pale/yellow
Odor	n.d*
Turbidity (NTU)	0.34
pH	7.5
Temperature (°C)	25
Conductivity (mS cm^{-1})	2.4
Total dissolved carbon (mg L^{-1})	43.8
Dissolved inorganic carbon (mg L^{-1})	29.7
Dissolved organic carbon (mg L^{-1})	14.1
Chemical oxygen demand (mg L^{-1})	21.0
Absorbance at 254 nm (AU)	0.215
Transmittance at 254 nm (%)	61
Total suspended solids (mg L^{-1})	20.5
Volatile suspended solids (mg L^{-1})	16.0
Sulfate (mg SO_4^{2-} L^{-1})	339
Chloride (mg Cl^- L^{-1})	308
Nitrite (mg NO_2^--N L^{-1})	< 0.3
Nitrate (mg NO_3^--N L^{-1})	28
Total nitrogen (mg N L^{-1})	52
Phosphate (mg PO_4^{3-} L^{-1})	< 1.3
Total Phosphorous (mg P L^{-1})	2.0

5.3.2. Analytical procedure

An HPLC (LaChrom Elite system from Merck-Hitachi equipped with a L-2130 pump and a diode array detector (L-2455 DAD)) was used for the quantification of E2 and EE2. A Purospher® STARR RP-18 (5 μm) (125 mm × 4 mm) reverse phase column was operated at 30 ºC with an L-2300 column oven. The equipment was operated in a gradient mode using acetonitrile and ultrapure water as mobile phases A and B, respectively: t = 0 - 6 min (40:60), t = 6 - 9 min (50:50), t = 9 - 13 min (30:70), t = 13 -

15 min (40:60) for A and B, respectively. The flow rate was set at 0.9 mL min^{-1}. Samples of 50 μL were injected with an L-2200 autosampler and the DAD was set at 280 nm.

H_2O_2 concentration was determined by the colorimetric metavanadate method (λ = 450 nm) (NOGUEIRA, OLIVEIRA, *et al.*, 2005). Total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD), total phosphorous, dissolved organic carbon (DOC), pH, temperature and turbidity and inorganic anions concentrations were determined according to Moreira et al. (2016).

5.3.3. Lab-scale tube-in-tube membrane reactor

The lab-scale tube-in-tube reactor comprises an inner ceramic ultrafiltration membrane (γ-Al_2O_3 from Inopor GmbH; pore size = 10 nm; cut-off = 20 kDa; porosity = 30-55%; Øexternal = 2.03 cm; Øinternal = 1.55 cm; total length = 20 cm; illuminated length = 17.4 cm) and an outer quartz tube, vertically fixed in a stainless steel structure. The membrane and quartz tube ends are firmly sealed by mobile polypropylene flanges. Technical characteristics and operational parameters of the reactor were presented by Vilar et al. (2020).

The annular reaction zone (ARZ) of the photoreactor was fed from a 5 L-jacketed vessel using a gear pump (model BVP-Z from Ismatec) and the radiation source was four UVC lamps (Puritec HNS G5 from Osram, λmax = 254 nm; nominal power = 6 W; useful power = 1.7 W) or four UVA lamps (TL 6W BLB 1FM/10X25CC from Philips, λmax = 365 nm; nominal power = 6 W; useful power = 0.7 W), located externally to the quartz tube. An aluminium shell around the photoreactor was used to avoid the direct eye exposure with light.

The catalyst was deposited on the ceramic membrane shell-side by dip-coating (Dip-coater RDC 15, Bungard Electronic GmbH). The membrane ends were previously capped in order to avoid the coating on the inner surface of the membrane. For the deposition procedure, a photocatalyst aqueous suspension (2 wt.% of TiO_2-P25 powder; two drops of TritonTM X-100, 500 mL) was prepared and magnetically stirred for 24 h. Then, the suspension was sonicated for 15 min at 50 kHz to promote the particles dispersion. The membrane was immersed on the TiO_2-P25 suspension (2% w/w) at an immersion and withdrawn velocity of 50 mm min^{-1}, with a dipping time of 1 min. After each deposition, the membrane was dried in the oven at 100 ºC during 15 min. The dip-coating process was repeated (1–9 times) until achieving the required amount of catalyst

immobilized on the membrane. Finally, the membrane was calcined in a furnace at a temperature gradient of 1 ºC min^{-1} from room temperature to 300 ºC with a dwell time of 3 h after 150 ºC and after 300 ºC. Then, the photocatalytic membrane was assembled on the reactor and cleaned by water permeation.

5.3.4. Experimental procedure

The ceramic tubular membrane was internally filled with H_2O_2 solution ($[H_2O_2]_{stock\ solution}$ = 5 g L^{-1} for PW tests), using an syringe pump (Nexus 6000 from Chemyx Inc.), with the back pressure regulator (BPR) fully open. Then, the BPR was fully closed and the H_2O_2 solution was gradually injected using the syringe pump, until some drops started to appear in the membrane shell side. At that point, the membrane pores were completely filled with the oxidant solution.

Subsequently, the membrane shell side was cleaned by pumping pure water through the ARZ. Later, 5 L of EDC solution ($[E2]_{inlet}$ = $[EE2]_{inlet}$ = 100 μg L^{-1}; T = 25 ºC) was pumped ($Q_{inlet,EDC}$ = 40 L h^{-1}; Re = 2056) through the ARZ while the syringe pump injected the H_2O_2 solution at low dosing rates and UVC or UVA lamps were turned on. Once the steady state was reached, samples were taken at selected times and then analyzed for EDCs and H_2O_2 (when used). The selected Reynolds number induces a helical motion of the water around the membrane shell-side, with a good degree of mixing as reported in a previous work (VILAR, ALFONSO-MUNIOZGUREN, *et al.*, 2020).

First, oxidant permeation tests, for the membranes with and without TiO_2-P25 coating, were performed in the absence of reaction (without EDC and light) for different oxidant dosing rates (0.7, 1.4, 2.0 and 2.7 mL min^{-1}) to evaluate the H_2O_2 concentration in the reactor outlet. After, EDC oxidation tests, using the synthetic EDC solution and H_2O_2 permeation method, were carried out using the membrane without catalyst (direct photolysis-without H_2O_2 permeation, only with H_2O_2-absence of UV light, UVC/H_2O_2 and UVA/H_2O_2) and with catalyst (UVC/TiO_2, UVA/TiO_2, UVC/H_2O_2/TiO_2 and UVA/H_2O_2/TiO_2). The effect of the catalyst film-layers number (3, 6 and 9 films), on the membrane shell side, was evaluated for the EDCs oxidation using the UVC/H_2O_2/TiO_2 system. The best nano-engineered membrane (NEM) was used in the remaining tests. The photoactivity of the NEM was always evaluated after three consecutive assays by repeating the first experiment. The NEM performance remained stable at the end of all tests.

Assays with an urban wastewater sample were performed using the best photocatalytic membrane. H_2O_2 stock solutions of 5, 10 and 15 g L^{-1} were used according to the oxidant dose required for each test (oxidant dosing rate of 2.7 mL min^{-1}). Direct injection of oxidant upstream of the reactor inlet was carried out to evaluate the system performance and compare with the permeation dosing method. The experimental conditions for all tests are presented in Table 5.2. After each experiment, the photoreactor was washed by pumping pure water through the ARZ.

5.4. Results and Discussion

5.4.1. H_2O_2 permeation tests

Initially, the oxidant concentration in the water stream at the outlet of the reactor was assessed in the absence of reaction (without EDC and light) for different H_2O_2 dosing rates (0.7, 1.4, 2.0, 2.7 mL min^{-1}) (Figure 5.1a).

H_2O_2 concentration increases with time up to steady-state conditions, which occur after $t/\tau \geq 90$ (τ is the space-time inside the illuminated ARZ). The horizontally dotted lines in Figure 5.1 represent the theoretical H_2O_2 concentration values considering the oxidant dosing rate, concentration of the oxidant stock solution and water flow. Figure 5.1a shows that the experimental H_2O_2 concentrations at steady state are very close to the theoretical ones.

Similar oxidant profiles were obtained for NEM (Figure 5.1b), which indicates that the TiO_2-P25 particles immobilized on the membrane shell side do not cause any type of pores obstruction. In fact, the inside/out permeation membrane presents a macroporous ceramic support on the shell-side, made out of α-Al_2O_3. Therefore, the TiO_2-P25 nanoparticles immobilized in the membrane macroporous ceramic support do not affect the membrane permeability.

Table 5.2 – Experimental conditions employed in all tests.

Water Matrix	$[H_2O_2]_{stock\ solution}$ (g L^{-1})	H_2O_2 dosing rate (mL min^{-1})	$[H_2O_2]_{ARZ}$ (mg L^{-1})	Radiation Source	TiO_2-P25 film layers	TiO_2-P25 mass (mg)	$[H_2O_2]_{Residual}$ (mg L^{-1})	E2 Conversion (%)	EE2 Conversion (%)
H_2O_2 Radial addition									
Synthetic EDC solution (PW)	-	-	-	-	-	-	-	3.7 ± 0.1	2.4 ± 0.5
	-	-	-	UVC	-	-	-	11.8 ± 0.3	9.6 ± 0.4
	5	0.7	5	UVC	-	-	3.4 ± 0.1	15.2 ± 0.8	14 ± 1
	5	1.4	10	UVC	-	-	8.5 ± 0.1	21 ± 1	20 ± 2
	5	2.0	15	UVC	-	-	13.7 ± 0.2	34 ± 4	31 ± 1
	5	2.7	20	UVC	-	-	18.4 ± 0.3	43.0 ± 0.8	39 ± 1
	-	-	-	-	3	17	-	4.4 ± 0.1	2.8 ± 0.3
	-	-	-	UVC	3	17	-	13.9 ± 0.1	14.4 ± 0.2
	5	0.7	5	UVC	3	17	3.1 ± 0.1	26 ± 2	24 ±
	5	1.4	10	UVC	3	17	7.3 ± 0.5	37 ± 2	30.9 ± 0.1
	5	2.0	15	UVC	3	17	12.0 ± 0.2	46.5 ± 0.1	43 ± 1
	5	2.7	20	UVC	3	17	17.3 ± 0.2	50.0 ± 0.1	43 ± 2
	-	-	-	UVC	6	21	-	27 ± 3	15 ± 2
	5	0.7	5	UVC	6	21	3.1 ± 0.1	33 ± 1	28.0 ± 0.5
	5	1.4	10	UVC	6	21	7.0 ± 0.2	39 ± 2	37 ± 4
	5	2.0	15	UVC	6	21	11.1 ± 0.1	50 ± 3	46.6 ± 0.4
	5	2.7	20	UVC	6	21	15.7 ± 0.2	51 ± 5	46 ± 4
	-	-	-	UVC	9	24	-	28.9 ± 0.7	22.2 ± 0.8
	5	0.7	5	UVC	9	24	2.6 ± 0.3	34 ± 2	30.9 ± 0.8
	5	1.4	10	UVC	9	24	6.9 ± 0.2	42.4 ± 0.1	41.0 ± 0.1
	5	2.0	15	UVC	9	24	11.5 ± 0.1	51 ± 5	48 ± 2
	5	2.7	20	UVC	9	24	14.5 ± 0.2	51 ± 2	48.0 ± 0.8

Water Matrix	$[H_2O_2]_{stock\ solution}$ (g L^{-1})	H_2O_2 dosing rate (mL min^{-1})	$[H_2O_2]_{ARZ}$ (mg L^{-1})	Radiation Source	TiO_2-P25 Film layers	TiO_2-P25 mass (mg)	$[H_2O_2]_{Residual}$ (mg L^{-1})	E2 Conversion (%)	EE2 Conversion (%)
H_2O_2 Radial addition									
Synthetic EDC solution (PW)	-	-	-	UVA	-	-	-	6.4 ± 0.3	4.2 ± 0.1
	5	0.7	5	UVA	-	-	3.4 ± 0.6	8.4 ± 0.8	6.9 ± 0.3
	5	1.4	10	UVA	-	-	9.2 ± 0.3	13.3 ± 0.6	10.3 ± 0.4
	5	2.0	15	UVA	-	-	14.3 ± 0.2	19.3 ± 0.5	15.2 ± 0.3
	5	2.7	20	UVA	-	-	18.4 ± 0.3	26.9 ± 0.3	19.5 ± 0.3
	-	-	-	UVA	9	24	-	17.0 ± 0.2	15.0 ± 0.1
	5	0.7	5	UVA	9	24	4.0 ± 0.1	19 ± 1	16 ± 1
	5	1.4	10	UVA	9	24	8.3 ± 0.5	21.6 ± 0.1	21 ± 1
	5	2.0	15	UVA	9	24	14.0 ± 0.6	25 ± 1	23.9 ± 0.8
	5	2.7	20	UVA	9	24	17.7 ± 0.3	35 ± 2	32 ± 2
Urban Wastewater (UWW)	5	2.7	20	UVC	9	24	18.2 ± 0.2	24 ± 1	23.4 ± 0.1
	10	2.7	40	UVC	9	24	34 ± 1	31.0 ± 0.1	29.7 ± 0.2
	20	2.7	80	UVC	9	24	72.8 ± 0.3	30.6 ± 0.1	29.6 ± 0.8
Injection of H_2O_2 upstream the reactor inlet									
Synthetic EDC solution (PW)	5	2.7	20	UVC	9	24	19.5 ± 0.2	34.2 ± 0.1	26 ± 2
Urban Wastewater UWW	10	2.7	40	UVC	9	24	39.0 ± 0.5	24.8 ± 0.7	19.2 ± 0.1

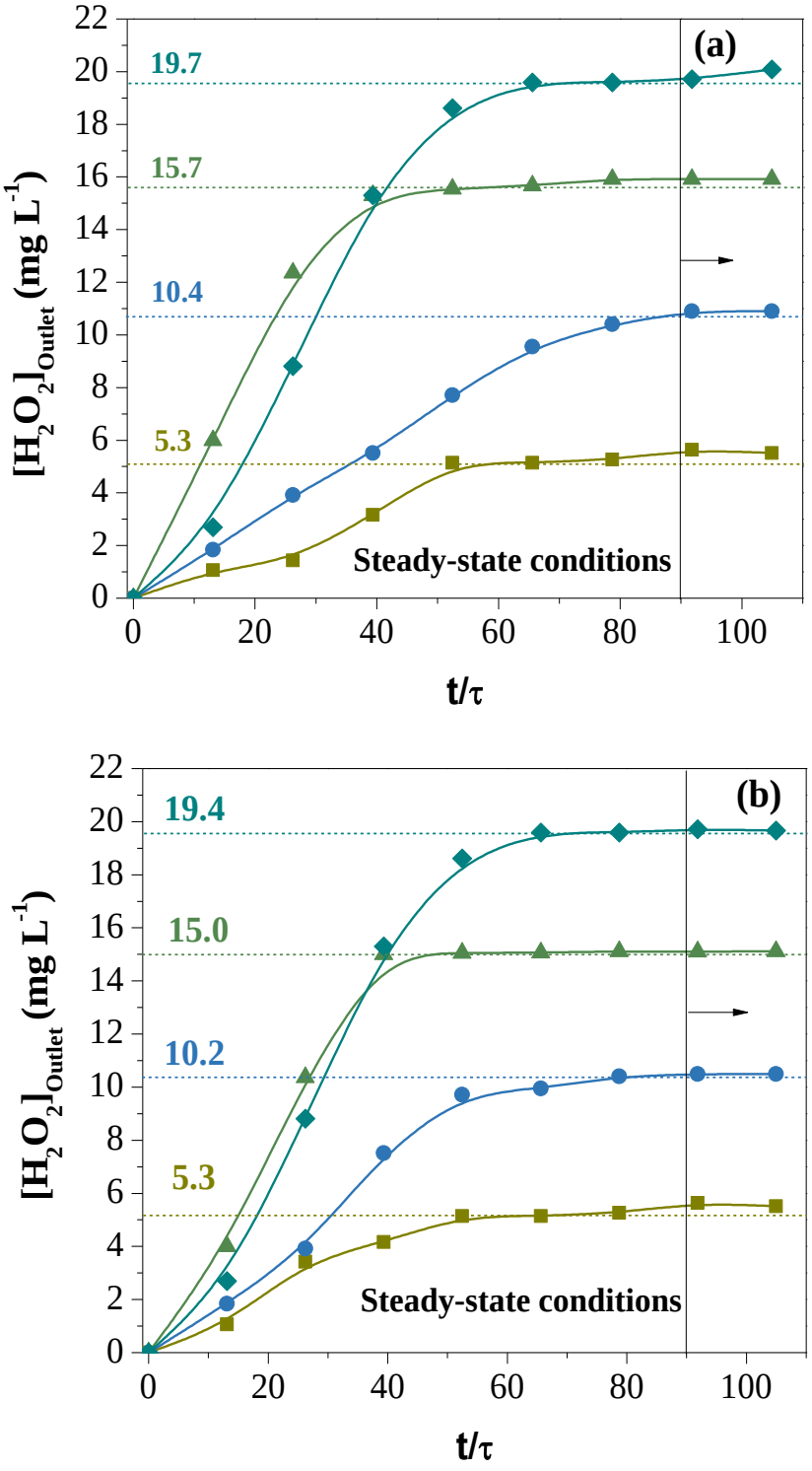
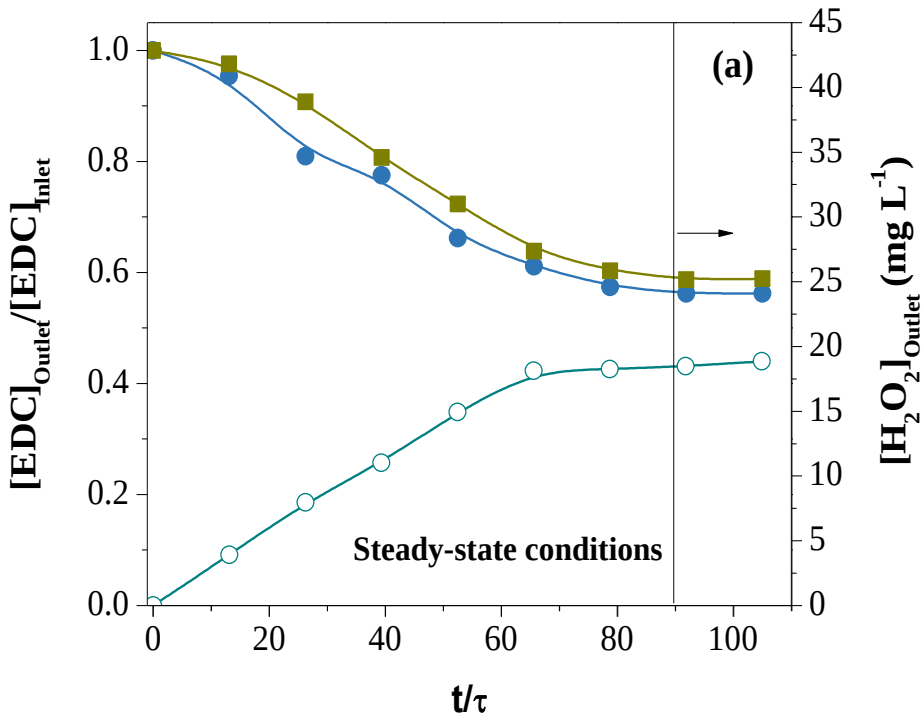


Figure 5.1 – H_2O_2 concentration profiles on the water flow side at the reactor outlet in the absence of UVC light and EDCs for different oxidant dosing rates (mL min^{-1}): 0.7 (■), 1.4 (●), 2.0 (▲), 2.7(♦); a) membrane without catalyst; b) membrane coated with 9 film-thin layers of catalyst.

Figure 5.2a,b shows the concentration profiles of EDCs at the outlet of the tube-in-tube reactor, considering an oxidant dosing rate of 2.7 mL min^{-1}, for the membrane with and without catalyst.

EDCs concentration at the reactor outlet reaches steady-state conditions after stabilisation of H_2O_2 concentration in the exit stream ($t/\tau \geq 90$). Therefore, in order to guarantee steady-state conditions, EDCs conversion for all assays was calculated by taking into account the EDCs concentration in the reactor outlet stream after $t/\tau \geq 100$, corresponding to an EDCs solution working volume of 5 L.

Figure 5.2a,b also displays the H_2O_2 concentration at the reactor outlet for the membrane with and without catalyst. At steady-state conditions, the H_2O_2 consumption for the membrane coated with catalyst was 2.5 times higher than in the absence of catalyst, indicating that H_2O_2 is also acting as acceptor of electrons from semiconductor conduction band, boosting the generation of hydroxyl radicals.



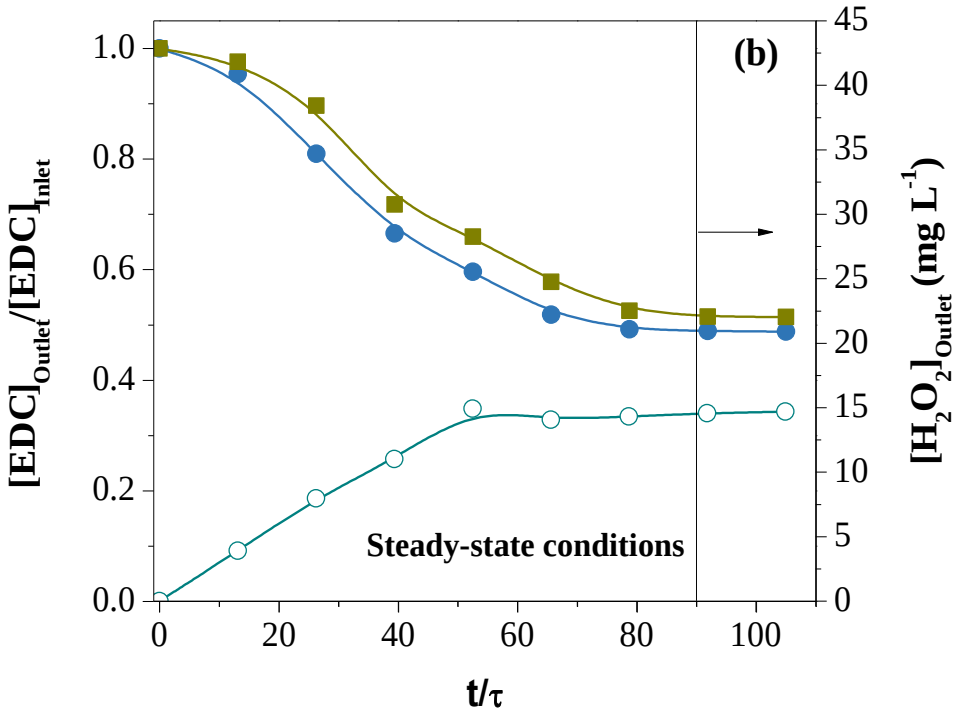


Figure 5.2 – E2 (●), EE2 (■) and residual H_2O_2 (○) concentration profiles on the water (PW) flow side at the reactor outlet in the presence of UVC light and with an oxidant dosing rate of 2.7 mL min^{-1}: a) membrane without catalyst; b) membrane coated with 9 film-thin layers of catalyst.

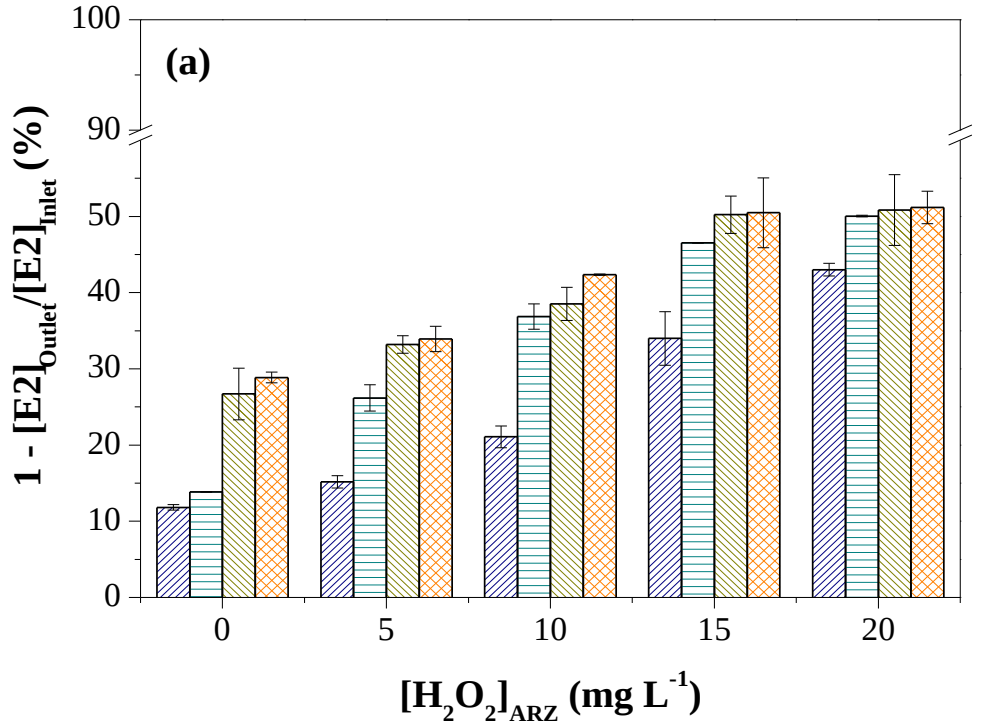
5.4.2. UVC/H_2O_2 (membrane without catalyst)

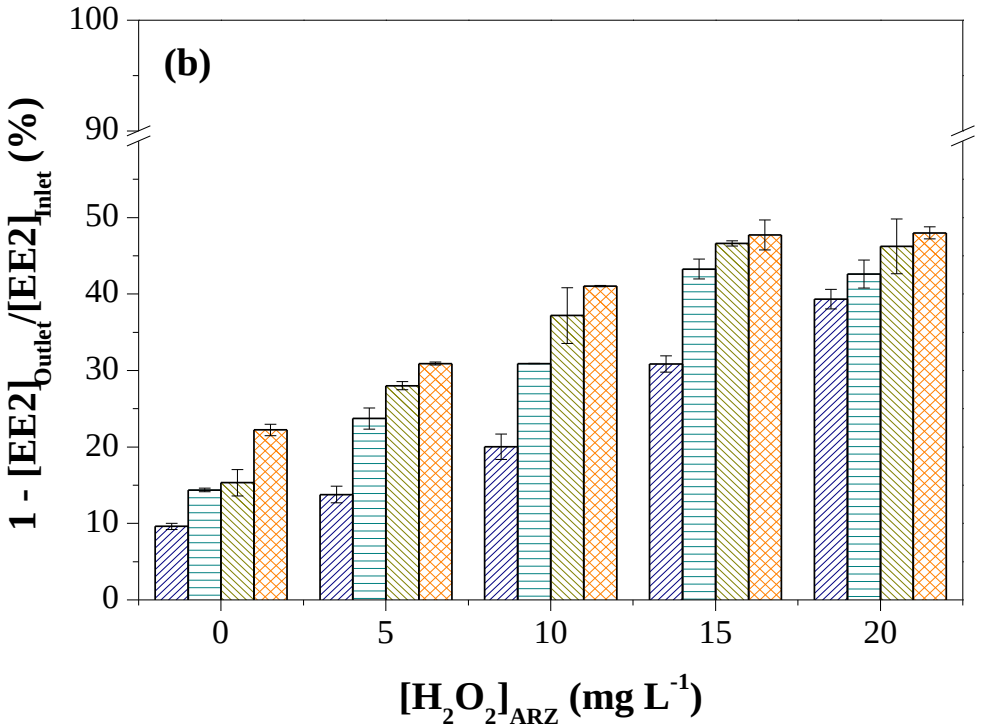
Preliminary assays showed that only UVC radiation didn't achieve an efficiently break down of the EDCs molecules, taking into consideration the low UVC fluence (45 mJ cm^{-2}) and low residence time inside the ARZ (4.6 s), reaching E2 and EE2 removal efficiencies of 11.8% and 9.6%, respectively (Figure 5.3a,b).

In the absence of UVC light, H_2O_2 was not able to oxidize efficiently EDCs molecules, even considering the highest H_2O_2 dose (20 mg L^{-1}) used in those tests (E2 and EE2 removal percentages of 8.1% and 5.9%, respectively).

However, coupling UVC radiation and H_2O_2 enhanced the EDCs oxidation efficiency, mainly resulting from the cleavage of H_2O_2 molecules into •OH radicals (Figure 5.3a,b) (PABLOS, MARUGÁN, *et al.*, 2013, MOREIRA, SOLER, *et al.*, 2016). EDCs removal increased with the increment of H_2O_2 dose, achieving a maximum efficiency of near 43% and 39% for E2 and EE2, respectively, for the highest oxidant dose (20 mg L^{-1}).

EE2 is a synthetic estrogen and presents a higher resistance to oxidation due to its chemical structure (SILVA, SALES, *et al.*, 2017). Higher amounts of oxidant were not tested in order to minimize the H_2O_2 concentration at the reactor outlet and reduce (or even avoid) the use of a subsequent treatment process to remove the residual H_2O_2.





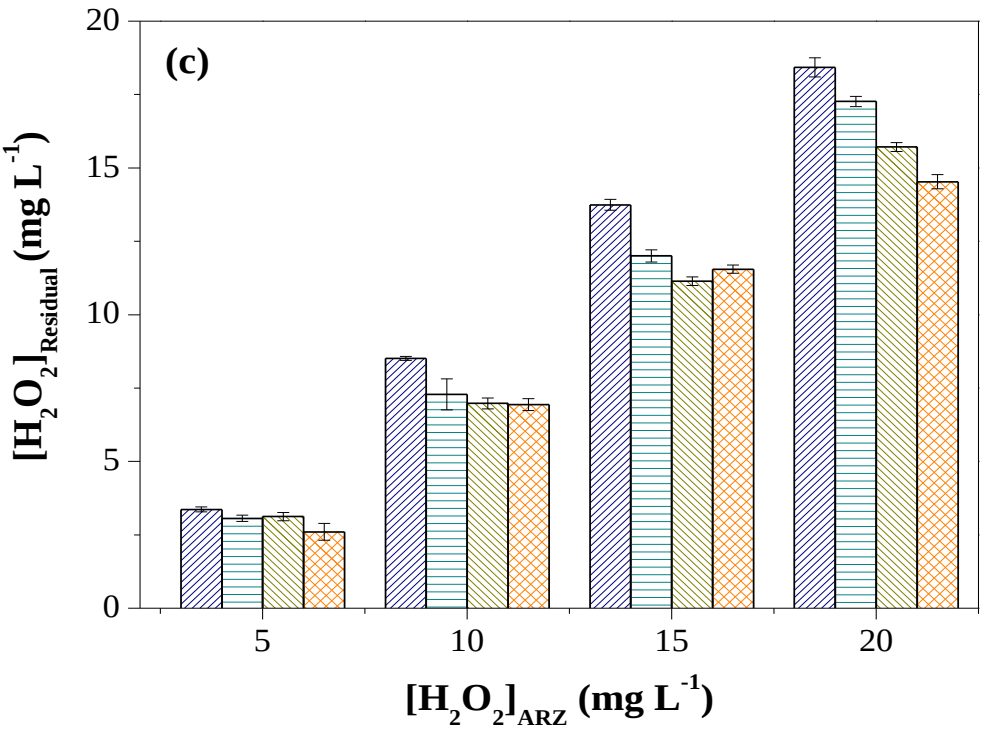


Figure 5.3 – Effect of H_2O_2 dosage and the number of catalyst films over the membrane shell side (3 - ▤, 6 - ▨ and 9 - ▩ TiO_2-P25 films layers; ▨ - no catalyst) on EDCs conversion from PW at steady-state conditions in the presence of UVC light: a) E2, b) EE2 and c) residual H_2O_2 concentration.

5.4.3. Nano-enhanced membrane

5.4.3.1. Effect of the amount of catalyst deposited on the membrane shell-side

The EDCs adsorption on the TiO_2 nanoparticles and on the membrane surface and, oxidation with only H_2O_2 at 20 mg L^{-1} (in absence of light) were in total below 10%, considering the low residence time (4.6 s) in the ARZ.

Figure 5.3a,b display a higher effect of the catalyst amount on the UVC/TiO_2 system, than for the UVC/H_2O_2/TiO_2, mainly for higher doses of H_2O_2, due to preponderance of UVC/H_2O_2 mechanism on EDC oxidation (CHEN, LI, *et al.*, 2001). However, EE2 shows a higher recalcitrant character than E2, mainly when using only TiO_2 photocatalysis (i.e. without H_2O_2 addition).

Nonetheless, the combination of the UVC/H_2O_2 photochemical process with UVC/H_2O_2/TiO_2 photocatalysis presented the best EDC conversions, near 50% for E2 and EE2, using the membrane coated with 9 layers of catalyst and an oxidant dose of 15 mg L^{-1}.

Although the use of higher amounts of oxidant lead to an improvement on EDCs conversion using the UVC/H_2O_2 photochemical process, a negligible effect was observed for the UVC/H_2O_2/TiO_2 system. The catalyst film immobilized in the membrane shell-side reduces the amount of oxidant required to achieve the same EDCs conversion using the UVC/H_2O_2 system.

The residual H_2O_2 concentrations for each condition are presented in Figure 5.3c. An increment in the amount of TiO_2-P25 immobilized onto the membrane shell-side increases the oxidant consumption, enhancing the production of hydroxyl radicals and therefore decreasing the residual H_2O_2 concentration. Considering the maximum EDCs removal efficiency at minimum residual H_2O_2 concentration, the membrane coated with 9 layers of TiO_2-P25 was selected to be used in the next set of experiments.

5.4.3.2. Effect of UV light wavelength

The EDCs conversion by UVA photolysis was two times lower than UVC photolysis which is in agreement with the EDCs absorption spectrum (Figure 5.4). Conversions are plotted in Figure 5.5a,b.

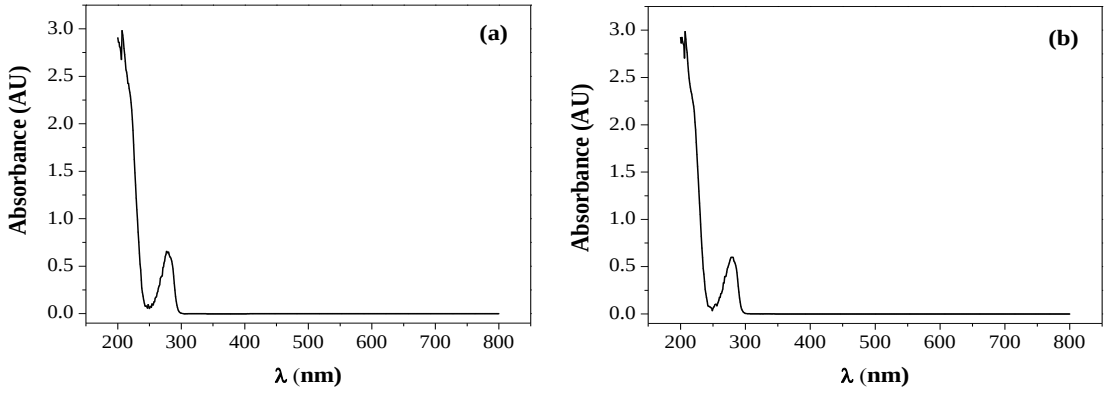
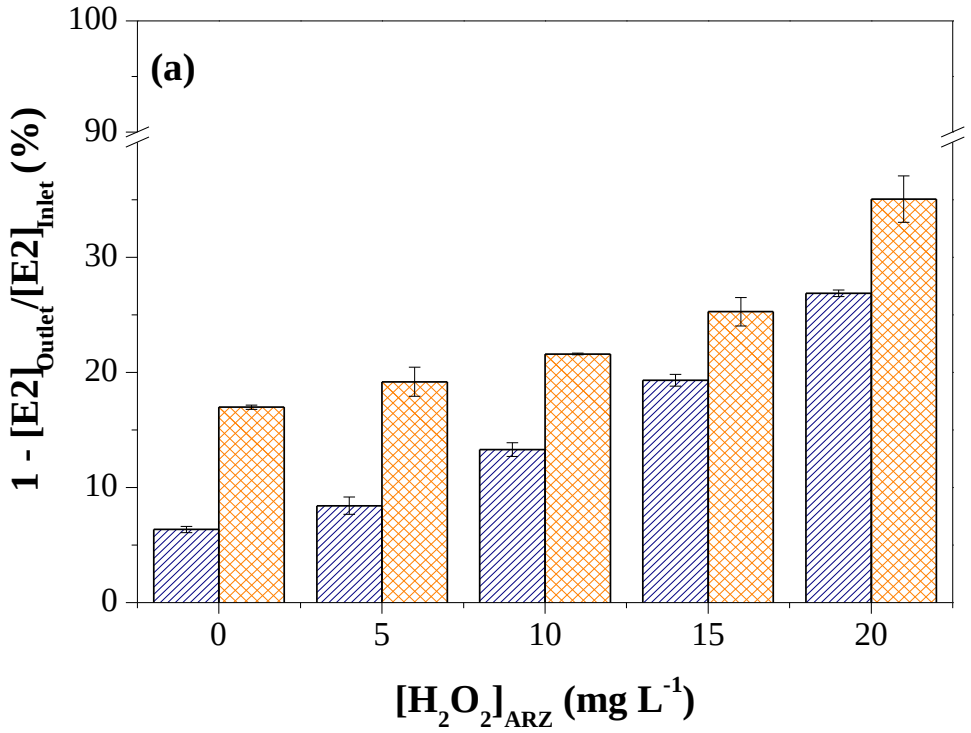


Figure 5.4 – Absorbance spectrum for the individual EDCs at a concentration of 10 mg L^{-1}: a) E2 and b) EE2.

Although the photolysis of hydrogen peroxide with UVA light presents a low efficiency, an increment on the oxidant dose using the membrane without catalyst, improved the EDCs conversion. This behavior can be also attributed to the decomposition of H_2O_2 by the membrane support, made out of α-Al_2O_3, and also to the oxidizing effect of H_2O_2 by itself. For the UVA/H_2O_2/TiO_2 system, using the membrane coated with 9 layers of TiO_2-P25, a continuous rise on EDCs conversion with the increment on oxidant dose was observed, resulting in conversions of 35% and 32% for E2 and EE2, respectively.



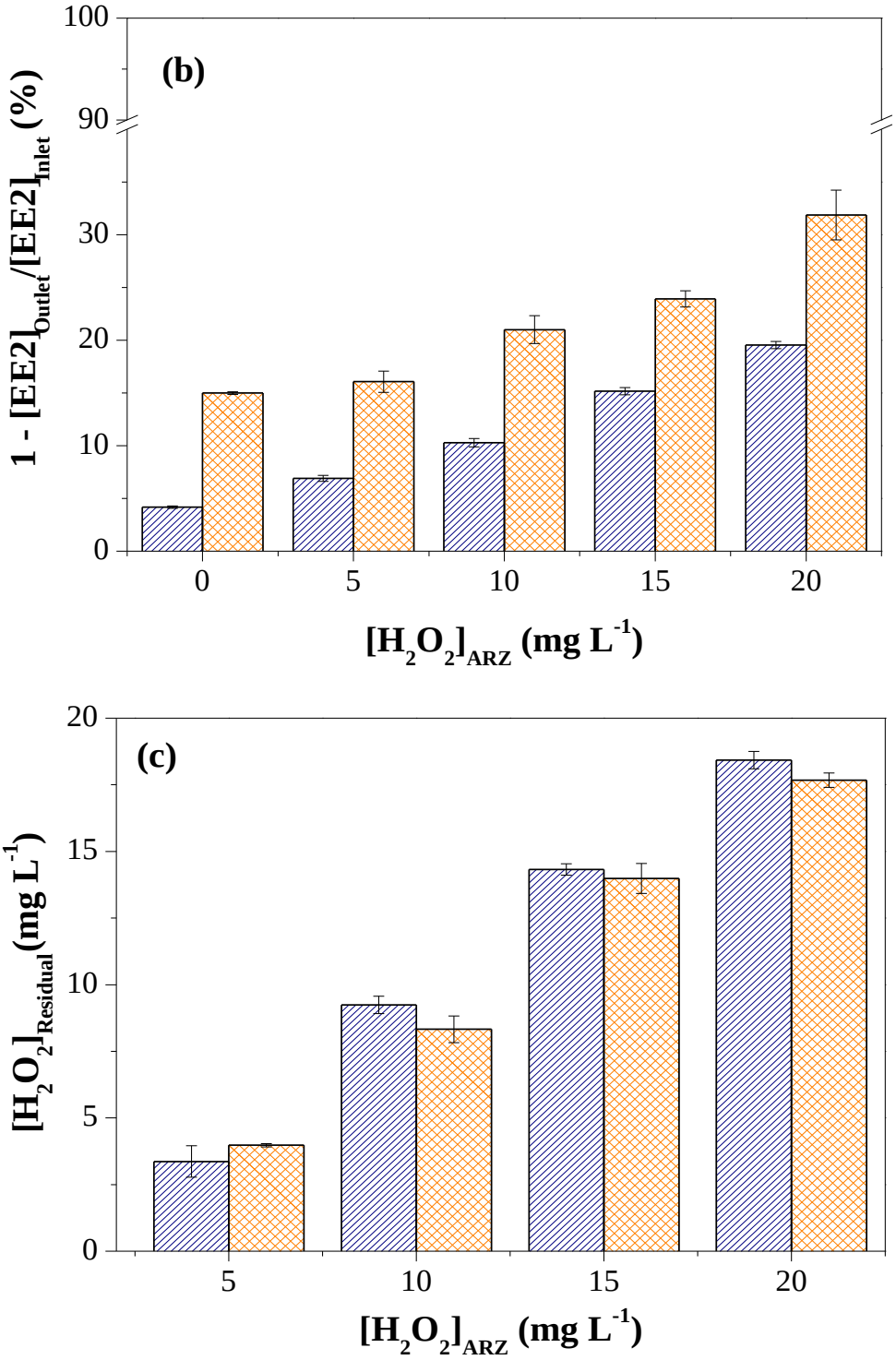
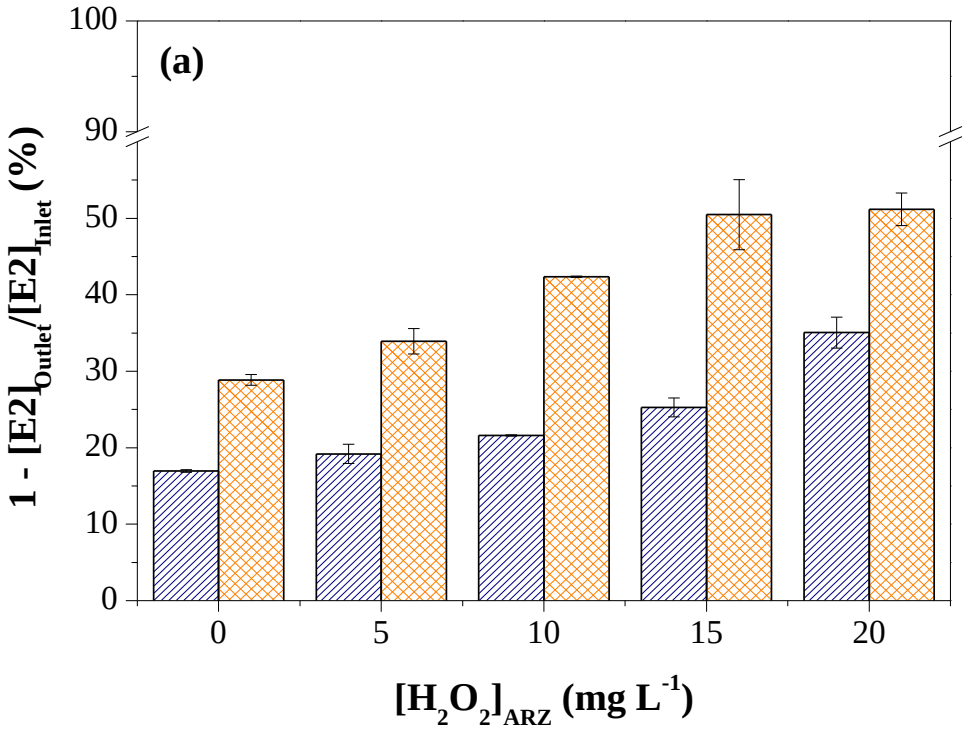


Figure 5.5 – Effect of H_2O_2 dosage and the presence/absence of catalyst over the membrane shell side (9 - TiO_2-P25 films layers; - no catalyst) on EDCs conversion from PW at steady-state conditions in the presence of UVA light: a) E2, b) EE2 and c) residual H_2O_2 concentration.

Figure 5.6a,b show that, for the membrane coated with the catalyst, the use of UVC light is more beneficial than the use of UVA light, which can be mainly attributed to two factors: i) a more efficient photolysis of H_2O_2 and EDCs in the annular reaction zone when using UVC light; ii) a higher UV power of the UVC lamp (1.7 W) when compared to the UVA lamp (0.7 W). This is also well correlated with the lower residual H_2O_2 concentration for the UVC/H_2O_2/TiO_2 system (Figure 5.6c), indicating a higher oxidant consumption.



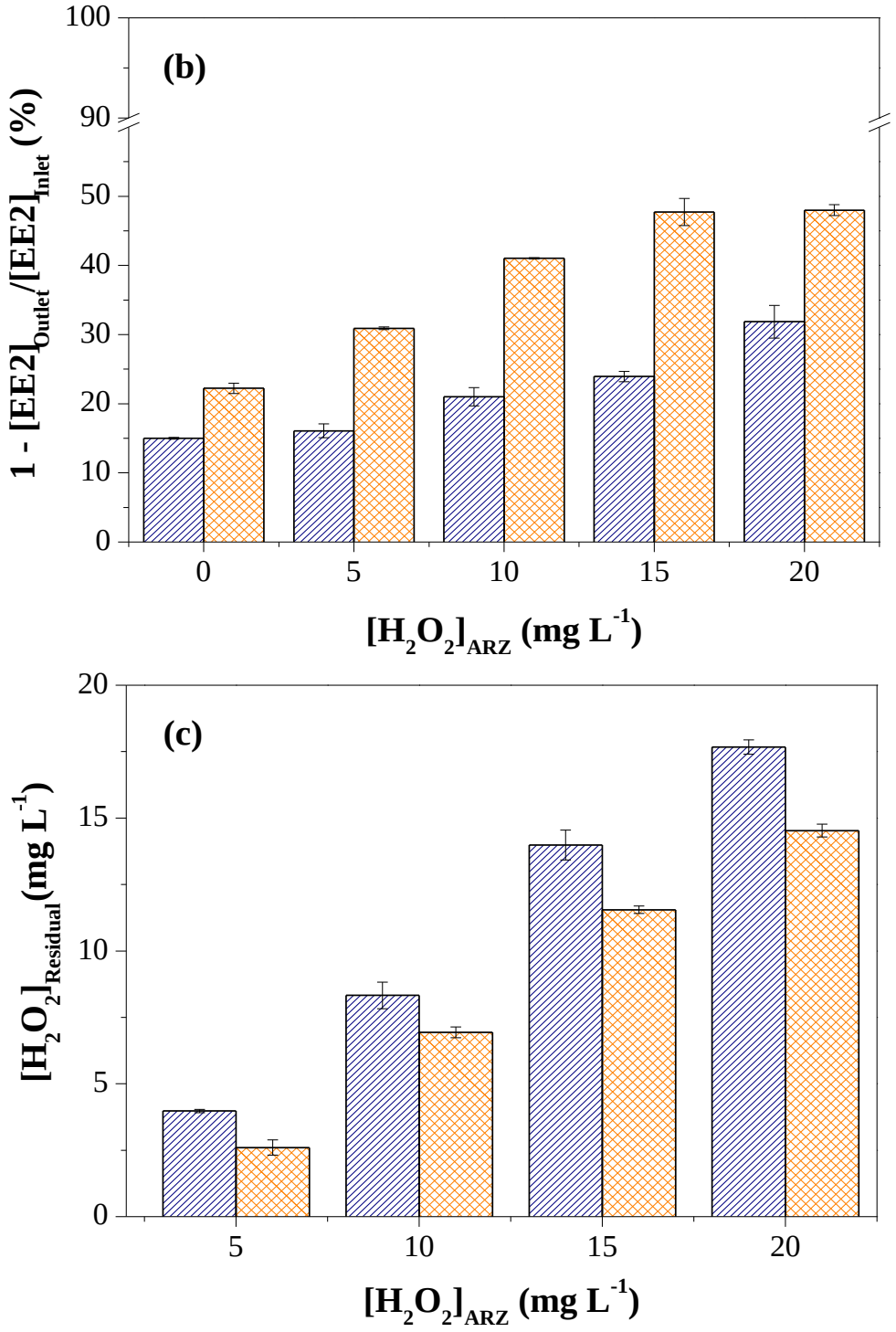


Figure 5.6 – Effect of light wavelength (- UVA; - UVC) on EDCs conversion from PW at steady-state conditions for different H_2O_2 dosages using the membrane coated with 9 layers of TiO_2-P25: a) E2, b) EE2 and c) residual H_2O_2 concentration.

5.4.3.3. Effect of the H_2O_2 dosage method

Figure 5.7 shows that the radial addition of H_2O_2 through unlimited number points across the membrane length enhances significantly the ability of UVC/H_2O_2/TiO_2 to remove EDCs (1.5 and 1.9 fold increase in E2 and EE2 removal, respectively), when compared with the injection of the same amount of oxidant upstream from the reactor inlet. This effect can be attributed to a more homogenous distribution of H_2O_2 molecules over the entire catalyst surface, when using the radial permeation method, improving its contact with the active catalyst sites. This is well correlated with the higher consumption of the oxidant for the radial addition method, reducing its residual concentration at the reactor outlet (Figure 5.7, inset plot).

This performance can be even boosted at full-scale, since the controlled oxidant "titration" to the catalyst surface will minimize the axial oxidant concentration profiles along the reactor, resulting in a uniform conversion rates over the reactor length.

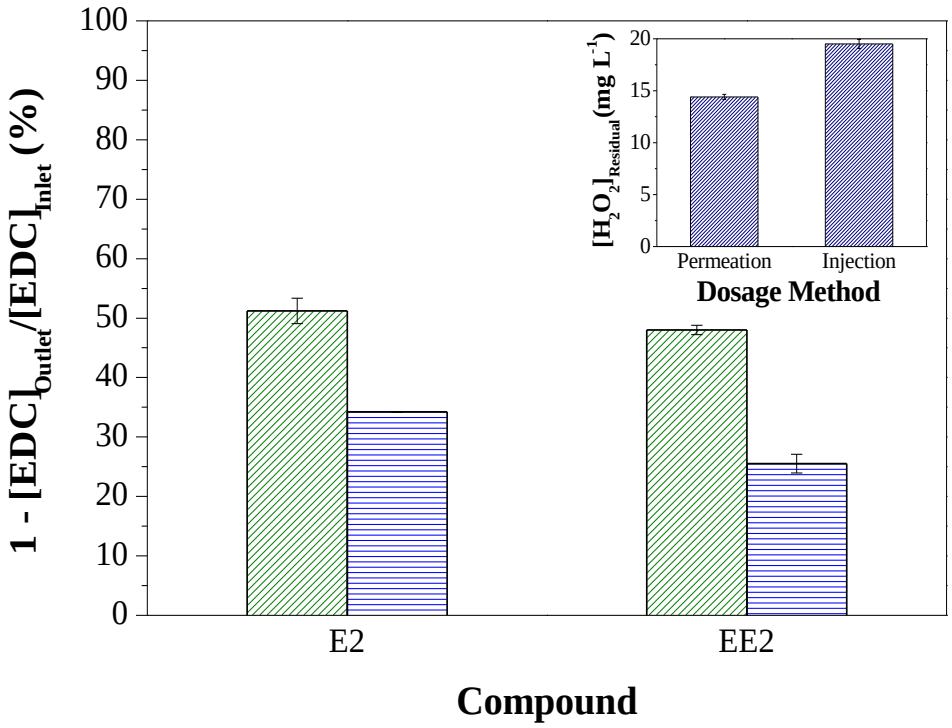


Figure 5.7 – Effect of oxidant dosage method on EDCs conversion from PW at steady-state conditions using the membrane coated with 9 layers of TiO_2-P25 in the presence of UVC light: ▨ - radial permeation; ▤ - injection upstream of the reactor inlet; $[H_2O_2]_{ARZ}$ = 20 mg L^{-1}.

5.4.3.4. Effect of water matrix

The EDCs removal using the tube-in-tube membrane photocatalytic reactor was also evaluated using the effluent from a municipal WWTP fortified with 100 µg L^{-1} of E2 and EE2. It is well known that organic and inorganic constituents present in urban wastewaters can induce UV light filtering effects and act as •OH scavengers, which decreases the process efficiency (WOLS, HOFMAN-CARIS, 2012, XIAO, YU, *et al.*, 2017).

The effect of carbonate and bicarbonate species (•OH scavengers) on the reactor performance was evaluated for the urban wastewater at different pH values (7.5, 7.0, 6.0 and 5.0), through the acidification with 4 M H_2SO_4.

Figure 5.8a shows a 2-fold increase on EDC removal efficiency with a decrease on inorganic carbon (IC) concentration from 29.7 mg L^{-1} (pH = 7.5) to 1.3 mg L^{-1} (pH = 5.0), indicating the strong effect of hydroxyl radicals on EDCs oxidation. As it was observed for the pure EDC solution, the radial addition of the oxidant results also in a higher EDC conversion for the urban wastewater matrix, when compared to the injection of the same amount of oxidant upstream of the reactor inlet (Figure 5.8b).

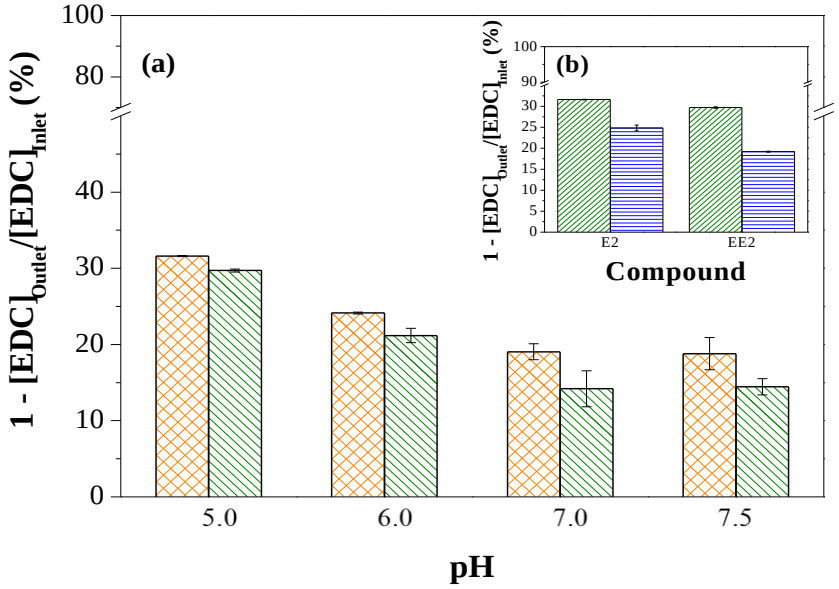


Figure 5.8 – a) Effect of UWW pH on EDCs conversion at steady-state conditions: - E2, - EE2; b) Effect of oxidant dosage method on EDCs conversion from UWW at steady-state conditions at pH 5.0: - radial permeation, - injection upstream of the reactor inlet; Conditions: membrane coated with 9 layers of TiO_2-P25; UVC light; $[H_2O_2]_{ARZ}$ = 40 mg L^{-1}.

Adding the same amount of oxidant (20 mg L^{-1}) to the ARZ, the urban wastewater matrix, with negligible concentration of carbonates and bicarbonates species (pH = 5.0), has a negative effect on reactor performance, resulting in a two-fold decrease on EDCs removal when comparing with the synthetic EDCs solution (Figure 5.9a vs Figure 5.3a,b). This mainly results from the presence of natural organic matter (NOM) containing light absorbing (low UVC transmittance) and reactive oxygen scavenger species (XIAO, YU, *et al.*, 2017, SANTOS, PAULISTA, *et al.*, 2019). Although Figure 5.9a shows an 8% increment on EDCs conversion when doubling the oxidant dose from 20 to 40 mg L^{-1}, the residual H_2O_2 concentration increased in a comparable extent.

A further increase on oxidant dose to 80 mg L^{-1}, resulted in a similar EDCs removal, mainly related to the low UVC fluence (45 mJ cm^{-2}) used in this work, which is quite lower than those existing in wastewater treatment plants (600-1000 mJ cm^{-2}) (JAMES, GERMAIN, *et al.*, 2014).

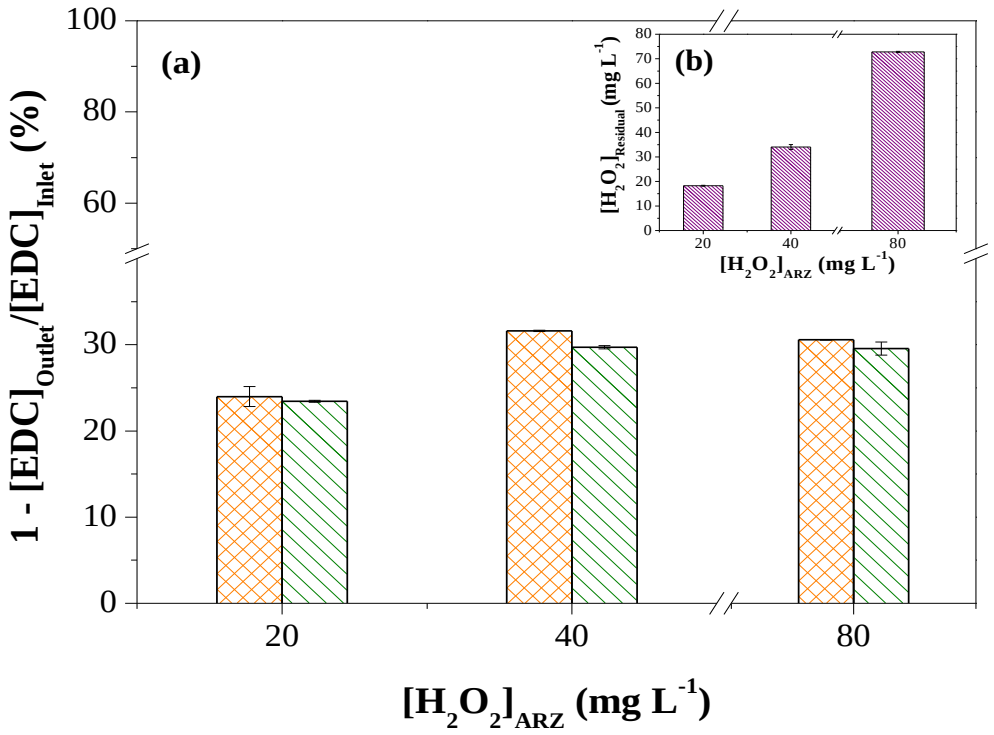


Figure 5.9 – Effect of H_2O_2 dosage on EDCs conversion from UWW at steady-state conditions using the membrane coated with 9 layers of TiO_2-P25 in the presence of UVC light: a) ▨ - E2; ▨ - EE2 removal; b) ▨ - residual H_2O_2 concentration.

5.5. Conclusions

A tube-in-tube membrane reactor boosted the efficiency of heterogeneous H_2O_2/TiO_2 photocatalysis for the removal of EDCs. The "virtually" unlimited number of H_2O_2 addition points along the membrane length allows a more homogenous distribution of H_2O_2 molecules over the catalyst immobilized in the membrane shell side, enhancing the production of hydroxyl radicals and therefore boosting the EDCs conversion and, minimizing the residual amount of oxidant in the outlet. The controlled oxidant "titration" to the catalyst surface ($UVC/H_2O_2/TiO_2$) resulted in a 1.5/1.4 and 1.9/1.7 fold increase on conversion of E2 and EE2, respectively, for PW/UWW matrices, when compared with the injection of the same amount of oxidant upstream of the reactor inlet. The helical movement of water around the membrane shell side increases the H_2O_2 radial dispersion, resulting in a more homogeneous distribution of the oxidant in the ARZ, enhancing its UVC photolytic cleavage into •OH, and reduces the number of local points near the membrane surface where greater oxidant concentrations are observed.

Maximum removal efficiencies of near 50% for both EDCs were obtained when using the membrane coated with 9 thin-film layers of TiO_2-P25, UVC light and H_2O_2 dosing rate of 2.7 mL min^{-1} (20 mg L^{-1}) for a synthetic wastewater, with a residual H_2O_2 concentration of 14 mg L^{-1}.

UWW matrix resulted in a 2-fold decrease on EDCs removal when compared with PW for the same oxidant dose, mainly resulting from organic and inorganic species, acting as UV filters and hydroxyl radical scavengers. However, EDCs removal efficiencies near 30% from UWW were obtained for an oxidant dose of 40 mg L^{-1}, with a low UV fluence (45 mJ cm^{-2}) and short residence time (4.6 s).

The tube-in-tube membrane configuration allows the efficient integration of membrane technology with TiO_2 photocatalysis and UVC/H_2O_2 photochemical systems in a single unit, maximizing the oxidant molecules distribution over the catalyst active sites and through the annular reaction zone.

6. FINAL CONCLUSIONS

The problem of the contaminants of emerging concern, mainly endocrine disrupting chemicals (EDCs), is an issue that draws the attention of environmental authorities worldwide, due to their negative impact on human health and aquatic organisms.

In this context, this thesis was intended to investigate two types of treatment aiming at the removal of both estrogens: 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). The innovative biological process of aerobic granular sludge (AGS), and the advanced oxidation process of heterogeneous photocatalysis in a membrane micro-reactor with radial permeation of H_2O_2, were investigated.

First, the effect of solids retention time (SRT) on the stability and performance of an AGS sequencing batch reactor (SBR) in terms of organic matter and nutrients removal was evaluated. The results showed that maintaining the SRT at 15 days, it was possible to achieve high COD (93%), ammonium (98%) and phosphorus (58%) removal efficiencies compared to that obtained at SRT of 20 and 30 days or even without controlling the sludge age. Besides, at this SRT, better physical characteristics of the aerobic granules were found, which directly impacted the process performance.

Once the reactor operation was stable, the study proceeded to evaluate the removal of EDCs in the AGS system. The physical properties of the aerobic granules were monitored after the estrogens addition and the performance in the combined removal of carbonaceous material, nitrogen, phosphorus and EDCs was evaluated. The AGS reactor showed satisfactory results in the simultaneous removal of nutrients and micropollutants, indicating that the microbial communities within the granules were able to adapt to the presence of EDCs. Overall removals of COD, ammonium nitrogen, and phosphorus reached 93%, 87% and 87%, respectively. On the other hand, E2 and EE2 removal was 99% and 93%, respectively. The main route of EDCs removal was biodegradation. Adsorption onto the granular biomass was found negligible (0.01% for E2 and 0.04% for EE2), while the percentage not removed was 0.93% and 7.1% for E2 and EE2, respectively. The results of the estrogenic activity of the analyzed samples showed that, although the high EDCs removal efficiencies achieved biologically, the residual concentrations detected in the effluent (reaching the maximum value of 0.122 μg L^{-1} of EQ-E2), can still cause adverse effects in the endocrine system of aquatic organisms.

Finally, the removal of 17β-estradiol and 17α-ethinylestradiol by heterogeneous photocatalysis showed that the best configuration tested was the combination of the processes: UVC/H_2O_2/TiO_2 using 9 thin-film layers of catalyst deposited on the nano-filtration membrane and a concentration of 20 mg L^{-1} of H_2O_2 permeated through the membrane of the micro-reactor. The maximum removal efficiencies for both E2 and EE2 reached approximately 50% for a synthetic matrix, using this setting, which enhanced the production of hydroxyl radicals and therefore boosted the EDCs conversion, minimizing the residual amount of oxidant in the outlet of the reactor. The integration of the membrane technology with photocatalysis and UVC/H_2O_2 photochemical processes in a single unit proved to maximize the oxidant molecules distribution over the catalyst active sites and allowed an efficient EDCs oxidation in a short residence time of 4.6 s.

In general, both individual processes studied in this thesis presented high removal efficiencies of pollutants and micropollutants. Based on their characteristics and applicability, a possible suggestion for combining the two processes for treatment of wastewater composed of organic matter, nitrogen and phosphorus and EDCs is proposed. First, an AGS SBR can be implemented for the abatement of the first three (simultaneous COD, N and P removal) and partial removal of the micropollutants, followed by a tertiary treatment using a membrane micro reactor in the best configuration found (UVC/H_2O_2/TiO_2) as a polishing step for complete removal of EDCs and other intermediate compounds resulting from the biological process. Such integrated approach may ensure a higher quality of the treated effluent to be subsequently discharged into water bodies, which is particularly relevant for countries where the levels of micropollutants in the outlet of wastewater treatment plants are controlled by environmental regulations.

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