

Universidade Federal do Rio de Janeiro

TREATMENT OF WASTEWATER CONTAINING ENDOCRINE
DISRUPTING COMPOUNDS BY AEROBIC GRANULES: ELUCIDATING
REMOVAL MECHANISMS AND MICROBIAL COMMUNITY
COMPOSITION

Cyntia Ely



Treatment of wastewater containing endocrine disrupting compounds by aerobic
granules: elucidating removal mechanisms and microbial community
composition

Cyntia Ely

Tese de Doutorado apresentada ao Programa de Pós-graduação em Engenharia Química, COPPE, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Engenharia Química.

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TRATAMENTO DE EFLUENTE CONTENDO COMPOSTOS DISRUPTORES
ENDÓCRINOS POR GRÂNULOS AERÓBIOS: ELUCIDANDO OS MECANISMOS
DE REMOÇÃO E COMPOSIÇÃO DA COMUNIDADE MICROBIANA

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Dezembro 2022

Orientadores: João Paulo Bassin

Márcia Walquíria de Carvalho Dezotti

Programa: Engenharia Química

A contaminação dos corpos hídricos por compostos desreguladores endócrinos (CDE) é preocupante. Esses podem interferir no sistema endócrino e causar efeitos adversos às comunidades aquáticas e, conseqüentemente, ao homem. Nesse sentido, dois estudos independentes foram conduzidos em laboratório visando avaliar o desempenho do processo de lodo granular aeróbio (LGA) na remoção de 17β -estradiol (E2), 17α -etinilestradiol (EE2) e bisfenol A (BPA) de efluentes domésticos simulados, sem e com o aporte adicional de sal ($12 \text{ g L}^{-1} \text{ NaCl}$). A contribuição dos mecanismos de adsorção e biodegradação nos compostos alvos, os efeitos no desempenho do sistema de grânulos aeróbios e a atividade e composição da comunidade microbiana frente à adição dos CDE foram avaliados. Em ambos os trabalhos, o E2 foi completamente biodegradado. BPA, no entanto, não foi prontamente metabolizado, mas sua remoção melhorou ao longo do tempo, sendo totalmente biodegradado devido à aclimação da biomassa e bioaumentação com bactérias degradadoras de CDE. O EE2 foi adsorvido na biomassa granular no período em que o reator foi mantido em anaerobiose, mas em concentração menor que os demais compostos. No período aerado, o EE2 foi desorvido e os microrganismos não conseguiram consumi-lo. A suplementação com CDE apresentou pouca influência no desempenho do reator em relação à remoção de matéria orgânica e amônio pelo processo de nitrificação. Em contrapartida, a atividade dos organismos

acumuladores de polifosfato foi afetada, provavelmente devido ao EE2. Além disso, observou-se uma alteração na comunidade microbiana ao longo da operação do reator. Os principais gêneros bacterianos foram diferentes para cada estudo, possivelmente devido à aclimação do LGA ter sido realizada sob concentrações salinas distintas.

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

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Dezembro 2022

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Department: Chemical Engineering

Contamination of water bodies by endocrine disrupting compounds (EDC) is a cause for concern. These can interfere with the endocrine system and cause adverse effects on aquatic communities and, consequently, on humans. In this sense, two independent laboratory studies were conducted to evaluate the performance of the aerobic granular sludge (AGS) process on removal of 17β -estradiol (E2), 17α -ethinylestradiol (EE2) and bisphenol A (BPA) from simulated domestic wastewater without and with the addition of salt ($12 \text{ g L}^{-1} \text{ NaCl}$) fed with simulated domestic wastewater and altered with EDC (E2, EE2 and BPA), one of them, saline effluent ($12 \text{ g L}^{-1} \text{ NaCl}$). The contribution of the adsorption and biodegradation mechanisms in the target compounds, the effects on the performance of the aerobic granules system and the activity of the microbial community against the addition of EDCs were evaluated. In both studies, E2 was completely biodegraded. BPA was not readily metabolized, but its removal improved over time, being fully biodegraded due to biomass acclimation and bioaugmentation with an EDCs-degrading bacteria. EE2 was sorbed onto the granular biomass in the anaerobic period, but at a lower concentration than the other compounds. In the aerated period, EE2 was desorbed and microorganisms were unable to consume it. The EDCs supplementation did not significantly affect the organic matter removal and ammonium by the nitrification

process. In contrast, the activity of polyphosphate accumulating organisms was affected, probably due to EE2. Moreover, the microbial community changed over time. The mainly bacterial genera were different for each study, possibly because the AGS acclimation was performed under different saline concentrations.

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LIST OF SYMBOLS

pKa	Acid dissociation constant
Log K _{ow}	Octanol-water partitioning coefficient
K _d	Partition of a compound between sludge and water
H	Shannon diversity index
E	Equitability index
D _{eq}	Equivalent diameter

LIST OF ABBREVIATIONS

AGS	Aerobic granular sludge
AMO	Ammonium monooxygenase
AND	Alternates of nitrification and denitrification
AOB	Ammonium-oxidizing bacteria
ATP	Adenosine triphosphate
BPA	Bisphenol-A
CAS	Conventional activated sludge
COD	Chemical oxygen demand
CPs	Chiral drugs
DGAOs	Denitrifiers glycogen accumulating organisms
DO	Dissolved oxygen
DPAOs	Denitrifying polyphosphate accumulating organisms
E1	Estrone
E2	17 β -Estradiol
E3	Estriol
EBPR	Enhanced biological phosphorus removal
EDCs	Endocrines disrupting compounds
EE2	17 α -ethinylestradiol
EPS	Extracellular polymeric substances
EU	European Union

F/M	Food to microorganisms ratio
FQs	Fluoroquinolones
GAOs	Glycogen accumulating organisms
H/D	Height to diameter ratio
HRT	Hydraulic retention time
NAS	Nitrifying activated sludge
NOB	Nitrite-oxidizing bacteria
PAOs	Polyphosphate accumulating organisms
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PN	Proteins
PPCPs	Pharmaceuticals and personal care products
PS	Polysaccharides
SBR	Sequencing batch reactor
SMX	Sulfamethoxazole
SND	Simultaneous nitrification-denitrification
SNDPR	Simultaneous nitrification, denitrification and phosphorus removal
SRT	Sludge retention time
USEPA	United States Environmental Protection Agency
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WWF	World Wide Fund for Nature
WWTPs	Wastewater treatment plants
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
COPPE	<i>Instituição Alberto Luis Coimbra de Pesquisa e Pós-graduação de Engenharia</i>
APHA	American Public Health Association
C/N	Carbon to nitrogen ratio
YES	Yeast Estrogen Screen
MBR	Membrane bioreactor
TSS	Total suspended solids
VSS	Volatile suspended solids

PBS	Phosphate-buffered saline
QIA	Quantitative image analysis
UPGMA	Unweighted pair group mean average
DGGE	Denaturing gradient gel electrophoresis
NGS	Next-generation sequencing
OUT	Operational taxonomic unit
HA	Humic acids
SNIS	<i>Sistema Nacional de Informações sobre Saneamento</i> (National system of information about sanitation)

1. INTRODUCTION

1.1 Contextualization and justification

The increasing worldwide consumption of chemicals has led to increased contamination of water resources by a new group of pollutants known as emerging contaminants. They are organic substances found in aquatic environments at very low concentrations, ranging from $\mu\text{g L}^{-1}$ to ng L^{-1} . Even under these conditions, these compounds have the potential to cause adverse effects on the health of organisms in water bodies (GARCIA-BECERRA; ORTIZ, 2018; STAMATIS; KONSTANTINO, 2013). Different groups of compounds are included in this category, such as pharmaceuticals and personal care products (PPCPs), endocrine disrupting compounds (EDCs) such as hormones, surfactants, chemicals used in various industrial branches, pesticides, etc. (LUO *et al.*, 2014).

EDCs have the potential or ability to alter the endocrine system functions. The natural estrogen, 17β -estradiol (E2), has a high estrogenic potential and is used as a standard (positive control) for measuring estrogenic activity (COLDHAM *et al.*, 1997). On the other hand, 17α -Ethinylestradiol (EE2) is a synthetic estrogen derived from E2 used in almost all modern formulations of oral contraceptive pills (ARIS *et al.*, 2014). Among the known xenoestrogens, bisphenol-A (BPA) has a wide range of industrial applications, making it one of the most commonly found in the environment (ZIELIŃSKA *et al.*, 2017; CYDZIK-KWIATKOWSKA *et al.*, 2017).

Previous studies have reported the biological effects of emerging contaminants, even at low concentrations. At a concentration of 4 ng L^{-1} , E2 resulted in the feminization of the fish *P. promelas*, and the same concentration of EE2 also caused the feminization of the fish *R. rutilus* (LANGE *et al.*, 2009; LÄNGE *et al.*, 2001). Another important aspect that should be considered is that these substances are generally found in aquatic ecosystems as mixtures and not as simple contaminants. Biochemical actions of some compounds can affect the actions of others, producing synergistic and antagonistic effects, which can become a complex ecological risk (QUADRA *et al.*, 2017).

These substances are introduced into the environment by various sources, such as domestic and industrial wastewater, which reach water bodies due to inadequate disposal and inefficiency of Wastewater Treatment Plants (WWTPs). In particular, developing countries, such as Brazil, still face serious problems related to environmental sanitation,

due to the lack of adequate policies that seek to invest in socio-environmental issues, such as, for example, the treatment and correct disposal of wastewater. In Brazil, many municipalities discharge domestic wastewater directly into water bodies, without any prior treatment. According to the National Sanitation Information System (SNIS, 2021), the volume effectively treated of the total generated is still low in the country, reaching only 51%. Furthermore, WWTPs are mostly designed to remove organic matter and nutrients using conventional biological processes. These classic approaches face a series of challenges derived mainly from the need to reduce the built-up area, consume less energy, produce less sludge and greenhouse gas emissions, and present resistance to biodegrade emerging compounds (AL-QAIM *et al.*, 2015; BOSHIR *et al.*, 2017).

The removal efficiency of emerging contaminants tends to be higher in tertiary treatment processes (AQUINO *et al.*, 2013). However, the implementation of tertiary treatment is often not feasible in developing countries due to the high costs involved. To overcome these issues and those related to the persistence and ecotoxicological effects of emerging compounds in the environment, it is necessary to improve the WWTPs seeking a better quality of treated effluent. In this context, there has been observed an increase in the application of biofilm processes to the detriment of conventional processes with suspended biomass. Biofilms allow the retention of high biomass concentration within the reactor and, consequently, greater capacity for pollutants removal. Several biofilm reactors have been developed in which the biomass is immobilized on a carrier that serves as support media.

A special case of biofilm composed of self-immobilized cells that do not require any support material, known as aerobic granular sludge (AGS), has been shown to be promising in the area of wastewater treatment (BASSIN, 2018). Aerobic granules are characterized by having a strong, dense, and regular structure, which gives the biomass good settling properties, high retention capacity in the system, and the ability to withstand high loads (LIU; TAY, 2004). The compact structure of the AGS leads to the formation of an oxygen diffusion gradient within these microbial aggregates, creating layers with aerobic, anoxic, and anaerobic zones (de KREUK *et al.*, 2005). Such stratification in the granular biomass allows different microbial communities involved in the combined removal of organic matter, nitrogen, and phosphorus to be present, allowing simultaneous conversions to occur in a single reactor. For this purpose, the reactor, normally a sequencing-batch reactor (SBR), is subjected to alternating aerobic and anaerobic periods.

Due to these intrinsic advantages, AGS can be applied as a promising alternative to increase the removal efficiency of EDCs. Due to the large amount of biomass that can be accumulated inside the reactor and the high microbial diversity harboured in such systems, the processes of biodegradation and biosorption can be intensified (SARMA *et al.*, 2016).

However, the ability to remove organic matter and nutrients can be compromised due to the presence of emerging contaminants, which may exhibit a complex and stable structure. Even at low concentrations, these compounds can affect the microbial community composition and, therefore, disrupt the performance of AGS processes (AMORIM *et al.*, 2018). For this reason, understanding the effect of the EDCs on the activity of important microbial functional groups and the physical properties of the granules is crucial for the implementation of AGS technology targeting the removal of these compounds. In this study, the target EDCs are E2, EE2, and BPA, often detected in domestic wastewater.

1.2 Objectives

Despite being a promising technology for biological wastewater treatment, there is still a place for expanding the knowledge about the AGS process. A deeper understanding of how the aerobic granular system will perform in the removal of the chosen emerging contaminants (E2, EE2 and BPA) and how the microbial community activity and diversity could act and contribute to improving effluent quality is necessary. Therefore, this doctorate thesis aimed at gaining further insights into the effects of emerging contaminants on the performance of AGS-based systems and elucidating the mechanisms of removal of the target compounds that occur in each period of the anaerobic-aerobic cycle of the reactor. Independent experimental lab-scale investigations were set up with AGS to treat EDCs-containing synthetic wastewater in Brazil and Portugal.

In the work development in Brazil, the ability of AGS to remove emerging contaminants (E2, BPA and EE2) was assessed. For that, the specific objectives were

- Quantify the compounds in both liquid and solid (granular biomass) phases;

- Analyze the contribution of adsorption and biodegradation mechanisms in each phase of an operating SBR cycle in the removal of the target compounds;
- Assess the effects of EDCs on the reactor performance parameters and AGS stability;
- Evaluate microbial community diversity and richness evolution over reactor operation under exposure to EDCs.

In Portugal, the same EDCs (E2, BPA, and EE2) were investigated, but with another approach, in synthetic wastewater under a saline environment (12 g L⁻¹ NaCl). For that. This study focused on:

- Improve the removal of EDCs (E2, BPA and EE2) by reactor bioaugmentation with a particular EDCs-degrading bacterial strain;
- Assess the contribution of adsorption and biodegradation mechanisms to the removal of the target compounds;
- Evaluate the effects of EDCs on reactor performance and biomass stability under a saline condition;
- Assess the composition and the diversity of the microbial community over time.

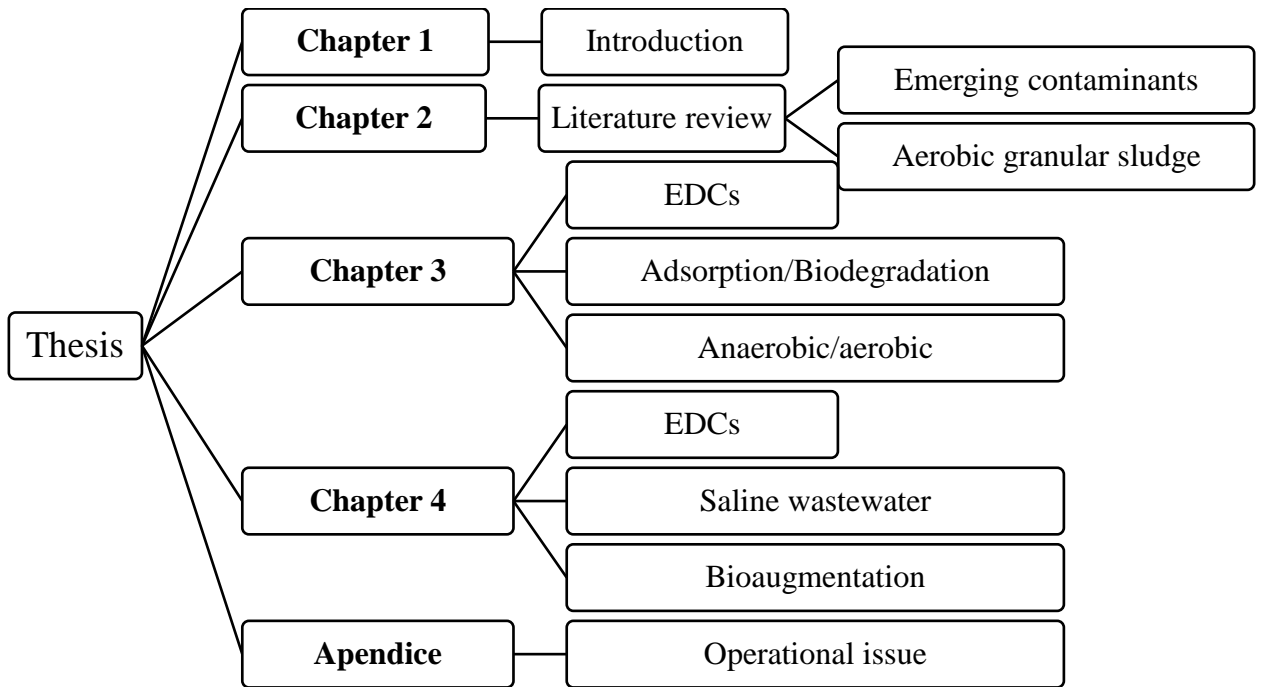
Before evaluating the removal of emerging contaminants, a separate study was conducted to understand and solve an operational issue with the reactor. This study will be presented in Apendice, as it is a complementary work not directly related to the main topic addressed in this thesis. Instabilities during the operation of aerobic granular systems are common and can harm system performance. As AGS systems depend on an anaerobic condition for the selection of certain bacterial groups that aid in keeping sludge stability, air leakage to the reactor during some periods may be detrimental to process operation.

Therefore, the following topics were addressed:

- Evaluate the effect of the air entrance during the anaerobic feeding on the AGS stability and performance on organic matter and nutrients removal;
- Propose strategies to overcome operating complications resulting from process disturbances, such as the emergence of filamentous organisms that cause system instability.

1.3 Thesis presentation

For a better understanding of the presentation of the thesis, below is illustrated a flowchart:



2. LITERATURE REVIEW

For the contextualization of this work, this chapter presents a brief literature review on the fundamental topics and concepts related to the research executed during this doctoral thesis. Emphasis was given to biological wastewater treatment and aerobic granular sludge technology. An overview of emerging contaminants and technologies applied for the biological treatment of these compounds is also given.

2.1 Emerging contaminants

Water is a valuable resource, crucial to all living organisms and for multiple human activities, such as domestic uses, agriculture, and industry. However, several emerging contaminants end up in aquatic compartments at concentrations between ng L^{-1} and $\mu\text{g L}^{-1}$. Even at low concentrations, these compounds harm the health of a living organism. Their effects are typically a result of chronic and not acute exposition (GARCIA-BECERRA; ORTIZ, 2018). Emerging contaminants can be natural or anthropogenic compounds such as pharmaceuticals and personal care products (PPCPs), endocrines disrupting compounds (EDCs) such as hormones, industrial compounds, pesticides, and others (LUO *et al.*, 2014).

These substances are produced and consumed all over the world, reaching the environment through domestic and industrial wastewater, runoff from agriculture, livestock, and landfill leachates. Several countries have legislation to regulate industrial wastewaters resulting from the production of PPCPs, pesticides, and others. However, regulations are still needed in other regions of the world (BARBOSA *et al.*, 2016). Agriculture is also an important source of emerging contaminants, mainly due to the use of pesticides to improve productivity and steroid hormones and antibiotics for livestock (BIRKETT; LESTER, 2002). The release of untreated wastewater directly into the water bodies or wastewater from municipal wastewater treatment plants (WWTPs) is another important route of emerging contaminants in the aquatic environment (TIJANI *et al.*, 2013). In fact, conventional WWTPs are often designed to remove organic matter and nutrients and not eliminate emerging contaminants (SANSON, 2012; TRAN *et al.*, 2018). In this context, the emerging contaminants can end up in surface water and groundwater aquifers, being consequently found in drinking water.

Overall, most emerging contaminants are not adequately regulated or monitored, and their risks are poorly understood. Bioaccumulation, chronic exposures, and possible interactions or synergistic effects with other organic compounds are among the issues related to these compounds (CARNEVALI *et al.*, 2018; VILELA *et al.*, 2018).

2.1.1 Endocrine disrupting compounds (EDCs)

In recent years, academia has demonstrated an increasing concern about EDCs because of their frequent detection in the effluent of WWTPs and aquatic environments (FRONTISTIS *et al.*, 2015). EDCs have emerged as a serious public health issue because they negatively affect the natural action of the endocrine system in humans and wildlife, such as synthesis, secretion, transport, and binding, altering the reproduction, metabolism, development, and behavior of living beings (USEPA, 1997). One of the main mechanisms of endocrine disruption is the linking of EDCs to the hormone receptors, which can inhibit or stimulate hormone metabolism or changes in hormone-binding proteins (CARNEVALI *et al.*, 2018).

Studies provide evidence that exposure to EDC causes adverse effects, which can lead to abnormal modulation or disruption of development and reproduction in aquatic life, especially fish (CABALLERO-GALLARDO *et al.*, 2016; CARNEVALI *et al.*, 2018). For example, KIDD *et al.* (2014) concluded that the chronic exposure of fathead minnow to 5 – 6 ng L⁻¹ of 17 α -ethinylestradiol (EE2) led to the feminization of male fish and altered oogenesis in females.

Within the EDCs, estrogens are the most commonly found in the aquatic environment. One of the major sources of EDCs is the residual from domestic wastewater (LUO *et al.*, 2014). Estrone (E1), 17 β -estradiol (E2), and estriol (E3) are natural estrogens, whereas EE2 is a synthetic hormone found most in oral contraceptives, both mainly excreted from humans. The concentration of natural estrogens in urinary excretion varies with age and gender, being different in premenopausal, pregnant, and postmenopausal women. The urinary excretion rate for premenopausal, pregnant, and postmenopausal women was found to be 10.73, 1,194, and 5 $\mu\text{g day}^{-1}$ for E1, 4.71, 347, and 2.78 $\mu\text{g day}^{-1}$ for E2, and 8.18, 24,078 and 2.78 $\mu\text{g day}^{-1}$ for E3, respectively. For men, it was 3.9, 1.5, and 1.5 $\mu\text{g day}^{-1}$, for E1, E2 and E3, respectively (LIU *et al.*, 2009). A review conducted by TING; PRAVEENA (2017) showed that the excretion rate for

women who used the synthetic steroid EE2 is $35 \mu\text{g day}^{-1}$, being 80% excreted as un-metabolized conjugates with 30% originating from feces and 22 to 50% from urine. Only 1 to 2% of the 80% EE2 excreted can be transformed into E1, E2 and E3. Therefore, the world's human population of about 7 billion discharges approximately $30,000 \text{ kg year}^{-1}$ of natural steroidal estrogens and an additional $700 \text{ kg of EE2 year}^{-1}$. However, the possible release of estrogens to the environment from livestock is much higher (ADEEL *et al.*, 2017).

In Brazil, GHISELLI (2006) detected in surface and potable waters E2 ($1.8\text{-}6 \mu\text{g L}^{-1}$), EE2 ($1\text{-}3.5 \mu\text{g L}^{-1}$), Bisphenol-A (BPA) ($2\text{-}64 \mu\text{g L}^{-1}$), along with other emerging contaminants. Similar concentrations of hormones were found in raw and treated wastewater samples, indicating that these compounds were not effectively removed in WWTPs. Moreover, MONTAGNER *et al.* (2019), in a study of 10 years of analyses of different samples (raw and treated sewage, surface, ground and drinking waters), carried out in the state of São Paulo (Brazil), identified a potential risk for aquatic life posed by EE2, E2, BPA, among others emerging contaminants in a preliminary risk assessment. For drinking water criteria, only E2 showed adverse effects on human health at the concentrations found in the studied scenario. In surface water samples collected from rivers in Rio de Janeiro before and after WWTP discharge, estrogens, including E2, were detected in all samples (DIAS *et al.*, 2015).

Due to the increasing concern about the EDCs, E2 and BPA compounds were included in the watch list of the European Union - Directive (EU) 2020/2184, which indicates a guidance value for each substance and compound harmful to health for water intended for human consumption (EUROPEAN COMMISSION, 2020). Although this regulation is very important and might be used as an example for other countries, most developing countries, such as Brazil, still do not have any regulations or official programs to prioritize or monitor EDCs.

A deeper literature review was carried out for the compounds evaluated in this work. It is presented in the following sections.

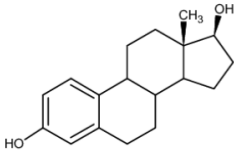
2.1.1.1 17β -Estradiol (E2)

17β -Estradiol (E2) is a natural sex hormone of the steroids class, produced by the ovarian follicles. It is responsible for breast growth and reproductive epithelia, maturation

of long bones, and development of secondary sexual characteristics. Furthermore, E2 is also responsible for maintaining the body tissues, ensuring skin elasticity and blood vessels, among other functions (BRUNTON *et al.*, 2005).

E2 is the most important biologically active and natural estrogen. In premenopausal women, E2 is the most abundant form of estrogen, but it varies throughout the menstrual cycle. The ovaries produce the estrogen E2, estrone (E1) is mainly derived from the adrenal androstenedione, and estriol (E3) is a metabolite of E1 and E2, biotransformed in the liver (NAZARI; SUJA, 2016).

Table 2.1 – Physicochemical properties of E2.

Chemical structure	Molecular formula	Molecular weight (g mol ⁻¹)	Solubility (mg L ⁻¹)	Log K _{ow}	pKa
	C ₁₈ H ₂₄ O ₂	272.4	13	3.94	10.6

In 2022, E2 is to be included in the first watch list because of its endocrine-disrupting properties and the risk they pose to human health. The guidance values of 1 ng L⁻¹ were established (EUROPEAN COMMISSION, 2022).

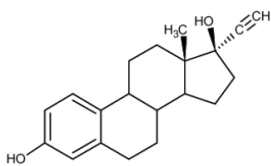
Livestock is one of the main sources of environmental contamination by E2, since this natural hormone is excreted by animals (SCHUH *et al.*, 2011). Considering the use of E2 for the treatment of some diseases, such as hormone replacement and some cancers, the excretion from its pharmaceutical use contributes to only 5% of the amount naturally excreted by living organisms (de MES; ZEEMAN; LETTINGA, 2005).

E2 is the most common and potent natural estrogen that causes estrogenic activity in wastewater. The exposure of aquatic organisms to this compound has raised concerns about the damage it causes to their health and the consequences for humans (NAZARI; SUJA, 2016). The clearest evidence was found in male fish swimming downstream of estrogen-impacted water sources (EE2-E2), even at low concentrations (1-2 ng L⁻¹). These fish showed sexual characteristics of both males and females, such as partially developed eggs or ovaries in the testes (WOODS; KUMAR, 2011).

2.1.1.2 17 α -Ethinylestradiol (EE2)

17 α -Ethinylestradiol (EE2) is a synthetic estrogen derived from E2 that is used in almost all modern formulations of oral contraceptive pills and is also commonly used in medications for the menopausal and postmenopausal syndrome, physiological replacement therapy, treatment of prostatic and breast cancer, and osteoporosis (ARIS *et al.*, 2014). Regarding the physicochemical properties, EE2 is a nonpolar compound with low solubility in water, low volatility, and is more resistant to biodegradation mainly due to the ethynyl group in the 17-position. As a moderately hydrophobic compound, EE2 binding to solids is favored (Table 2.2).

Table 2.2 – Physicochemical properties of EE2.

Chemical structure	Molecular Formula	Molecular weight (g mol ⁻¹)	Solubility (mg L ⁻¹)	Log K _{ow}	pKa
	C ₂₀ H ₂₂ O ₂	296.4	4.8	3.67	10.4

EE2 presents high biological activity due to its free trafficking through cell membranes and its molecular activity mechanism and may affect aquatic environments even at an extremely low concentration (ng L⁻¹) (MENASHE *et al.*, 2020). Previous studies have reported the ability of EE2 to affect the endocrine system of exposed organisms by altering sex determination, delaying sexual maturity, and decreasing secondary sexual characteristics (ARIS *et al.*, 2014).

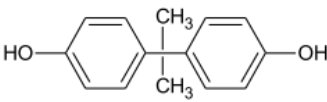
In humans, the binding affinity of EE2 to the estrogen receptor is one to two times higher than E2 and has been shown to be up to five times higher in some fish species (ARIS *et al.*, 2014). This higher receptor affinity indicates that EE2 can be a more potent estrogenic compound, being associated with 35-50% of estrogenicity in surface water and the remaining attributed to other estrogens (SEDIGHI *et al.*, 2019).

2.1.1.3 Bisphenol-A (BPA)

Bisphenol-A (2,2-bis-4-hydroxyphenylpropane) (BPA) presents a basic chemical structure consisting of two phenolic rings connected by acetone (Table 2.3). About three million tons of BPA are produced worldwide and used to manufacture polycarbonate plastic and epoxy resin present in most food and beverage cans (CORRE *et al.*, 2015). This compound is frequently found in CDs, sunglasses, plastic bottles, baby bottles, food packaging, etc. (ZÜHLKE *et al.*, 2016).

BPA is present in the environment because of the direct release of industrial wastewater from manufacturing or processing facilities and many diffuse sources, including municipal wastewater (ZIELIŃSKA *et al.*, 2014). According to Directive (EU) 2020/2184, maximum BPA concentration in water should be $2.5 \mu\text{g L}^{-1}$ (EUROPEAN COMMISSION, 2020). For the Directive (EU) 2018/213, a specific migration limit of $0.05 \text{ mg BPA kg food}^{-1}$ should be set for plastic materials and articles to ensure that exposure to BPA does not endanger human health (EUROPEAN COMMISSION, 2018).

Table 2.3 – Physicochemical properties of BPA.

Chemical structure	Molecular formula	Molecular weight (g mol ⁻¹)	Solubility (mg L ⁻¹)	Log K _{ow}	pKa
	C ₁₅ H ₁₆ O ₂	228.3	300	3.3	9.6 – 11.3

BPA has been classified as EDC by the United States Environmental Protection Agency (USEPA) and the World Wide Fund for Nature (WWF) and has been declared a global social and environmental issue (MOHAPATRA *et al.*, 2010). It is considered a xenoestrogen because it interacts with estrogen receptors and acts as an agonist or antagonist via estrogen receptor-dependent (SHAFEI *et al.*, 2018). Its affinity with the estrogen receptors is 10,000 to 100,000-fold weaker than E2, therefore, less potent (WELSHONS *et al.*, 2006). Although BPA has been associated with weak estrogenic activity, it has serious health effects in humans and wild animals. BPA exposure could induce a gain in body weight and an alteration of plasma triacylglycerol, total cholesterol, and glucose levels (CORRE *et al.*, 2015). Moreover, BPA has been shown to play a role

in the pathogenesis of various hormone-dependent tumors such as breast, ovarian, prostate, and other cancers (SHAFEI *et al.*, 2018).

2.2 Biological wastewater treatment

Biological treatment systems promote the degradation of organic and inorganic compounds in wastewater through the action of microorganisms. These consume the substrates present in the wastewater, forming inert products with low polluting potential (von SPERLING, 1996). It is important to remove organic matter and nutrients, not only ammonium nitrogen but also the oxidized nitrogen forms (nitrite and nitrate) and phosphorus, which cause negative impacts on the receiving bodies, such as eutrophication.

2.2.1 Biological removal of carbonaceous organic matter

The removal of organic matter from wastewater depends on processes such as adsorption, absorption, synthesis, and respiration. In general, the biological degradation of pollutants in wastewater is directly related to their biodegradability. In the first stages of treatment, organic compounds are adsorbed on the surface of biological flocs, films, or granules, where they are sequentially degraded by extracellular enzymes to form simpler molecules that can cross the bacterial cell wall. Then, this material is metabolized, which will provide the energy necessary for the cells to function (BASSIN; DEZOTTI, 2008).

Easily biodegradable substrates or products of adsorption and absorption processes are used by microorganisms for energy generation and cellular synthesis. The chemical transformation of the substrate into stable products and energy through a reaction in which organic matter is oxidized by an oxidizing agent present in the liquid is called catabolism (von SPERLING, 1996). In the process called anabolism, some reactions lead to the formation of cellular material using the energy produced in catabolism (van HAANDEL; MARAIS, 1999).

For the degradation of organic matter, heterotrophic microorganisms with different metabolism (aerobic, anoxic, anaerobic, or a combination of the previous) are used. In aerobic metabolism, microorganisms use oxygen as the final electron acceptor,

while nitrate or nitrite are used under anoxic conditions. In anaerobic digestion, facultative or strictly anaerobic bacteria degrade complex organic compounds, converting them into gases such as methane (60 to 70%), carbon dioxide (40 to 30%), and other mineralized by-products.

2.2.2 Biological nitrogen removal

Domestic wastewater shows in its composition nitrogen in organic form (urea, amino acids, and other organic substances within the amine group), ammoniacal form (free ammonia – NH_3 or ammonium ion – NH_4^+), and oxidized form (nitrite - NO_2^- or nitrate - NO_3^-). Nitrogen is biologically converted into nitrogen gases, inert and not aggressive, that can escape to the atmosphere by the processes known as nitrification and denitrification. Figure 2.1 illustrates nitrogen conversions describing the organisms capable of performing each step.

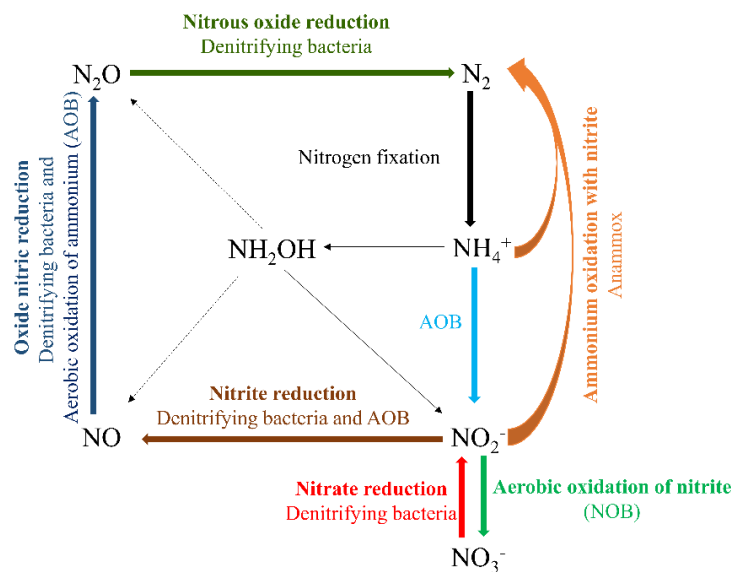


Figure 2.1 – Biological conversions of nitrogen and the microorganisms responsible for performing each step.

Source: Adapted from Kampschreur *et al.* (2009).

In the first step of nitrogen removal, during aerobic conditions, ammonium is oxidized to nitrite by ammonium-oxidizing bacteria (AOB). The most common AOB belong to the genera *Nitrosomonas* and *Nitrosospira* (Equation 2.1) (SCHRAMM *et al.*,

1998). Nitrite is then converted into nitrate by nitrite-oxidizing bacteria (NOB), such as *Nitrobacter* and *Nitrospira* (Equation 2.2) (SCHRAMM *et al.*, 1998; WARD *et al.*, 2011). This two-step process is called nitrification. The global equation is shown in 2.3.



In the subsequent step, denitrification occurs in an anoxic environment at low oxygen concentrations, where nitrate is reduced to nitrogen gas (N_2) by heterotrophic bacteria. Nitric oxide (NO) and nitrous oxide (N_2O) are possible intermediary products (METCALF; EDDY, 1991). Nitrate reduction to N_2 occurs in sequential steps (2.4) and is catalyzed by specific enzymes. Commonly, denitrification occurs as follows: nitrate is reduced to nitrite by nitrate reductase; nitrite is subsequently reduced to nitric oxide by nitrite reductase; nitric oxide is then reduced to nitrous oxide by the enzyme nitric oxide reductase; and finally, nitrous oxide is reduced to nitrogen gas by the enzyme nitrous oxide reductase (KAMPSCHREUR *et al.*, 2009).



2.2.3 Biological phosphorus removal

Wastewaters may contain phosphorus from detergents, urine, and feces. To prevent the eutrophication of water bodies, it is necessary to remove this nutrient in the WWTPs. Biological removal of phosphorus can be efficiently performed by the enhanced biological phosphorus removal (EBPR) process, which is performed by polyphosphate-accumulating organisms (PAOs). PAOs are relatively slow-growing organisms compared to other heterotrophic organisms, and their metabolism involves cycles of formation and consumption of intracellular polymers (polyphosphates, glycogen, and polyhydroxyalkanoates (PHA)) under alternating oxidation-reduction conditions (Van LOOSDRECHT *et al.*, 1997).

In an anaerobic environment, PAOs can metabolize easily biodegradable carbon sources, such as volatile fatty acids (VFA), and store them as intracellular polymers. If

acetate is the carbon source, the main polymer stored is polyhydroxybutyrate (PHB) (BASSIN, 2012). The energy in the form of adenosine triphosphate (ATP), required to metabolize VFA and transform them into PHA, is obtained by breaking down the polyphosphate chains and subsequently releasing the phosphate into the bulk liquid. Part of the energy also comes from the hydrolysis of intracellular glycogen, which also provides the reduction potential for converting VFA to PHA (MINO *et al.*, 1998).

In the subsequent aerobic phase, when the electron acceptor is present in the absence of enough amount of organic carbons, PAOs use the stored PHA as an energy source, and orthophosphate is absorbed from the bulk liquid for maintenance, growth, and glycogen and polyphosphate production. Orthophosphate is absorbed from the bulk liquid and synthesized as polyphosphate. Due to bacterial growth, phosphorus assimilation under aerobic conditions is higher than phosphorus release in the anaerobic phase, resulting in the effective removal of phosphorus from the liquid (MINO *et al.*, 1998). This way, phosphorus is removed from the treatment system by discarding excess sludge rich in polyphosphate (Van LOOSDRECHT *et al.*, 1997).

Phosphorus removal can also be achieved by microorganisms capable of denitrification in anaerobic-anoxic conditions. In this case, denitrifying polyphosphate-accumulating organisms (DPAOs) use nitrite and/or nitrate as electron acceptors (NANCHARAIAH; REDDY, 2018). Additionally, these microorganisms can store PHAs and use them as the electron source for denitrification.

Possible deterioration of the EBPR process can be caused by the competition for the available organic substrate between PAOs and glycogen-accumulating organisms (GAOs), which are also capable of proliferating in alternating anaerobic and aerobic conditions (CROCETTI *et al.*, 2002). GAOs have a metabolism similar to PAOs, as they also can anaerobically absorb VFA. However, these organisms use glycogen as an energy source, and they do not store polyphosphate. Therefore, the dominance of GAOs in the reactor is associated with a decrease in phosphorus removal (OEHMEN *et al.*, 2006; SAUNDERS *et al.*, 2003).

2.3 Biological removal of emerging contaminants

Some available technologies that are very effective in removing emerging contaminants from WWTPs include (IWA, 2010): advanced oxidative processes,

ultraviolet radiation, and adsorption processes (e.g., using activated carbon). However, these processes demand high investment and operating costs compared to biological treatment systems (AQUINO *et al.*, 2013; GARCIA-RODRÍGUEZ *et al.*, 2014). Considering that most WWTPs employ conventional wastewater treatment systems, which use biological reactors, the removal of emerging contaminants through its biodegradation has already been studied by several authors.

The emerging compounds can be present in a biological reactor in three phases (solid, liquid, and gas streams). Therefore, three main removal mechanisms can be distinguished: biotransformation, sorption, and volatilization. Volatilization is not discussed here because this mechanism is negligible for the overall removal of emerging contaminants (ALVARINO *et al.*, 2018).

In WWTPs, the fate and behavior of emerging contaminants depend on the physicochemical properties of the compound. The accumulation of compounds on solids is important due to the large concentration of biomass in biological reactors. Sorption depends on the lipophilic character or its tendency to be ionized or dissociated in an aqueous phase. In sludge, octanol-water partitioning coefficient ($\log K_{ow}$) and the acid dissociation constant (pK_a) determine their sorption trend. The solid–water distribution coefficient physicochemical (K_d) defines the ratio between the concentrations in the solid and liquid phases at equilibrium conditions, commonly used to determine the fraction sorbed onto sludge (SUÁREZ *et al.*, 2008; TRAN *et al.*, 2018).

Sorption occurs mainly by absorption and adsorption. The first involves hydrophobic interactions between the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of microorganisms, as well as the fat fractions of the sludge. On the other hand, the second involves electrostatic interactions of positively charged groups with negatively charged surfaces of microorganisms (TERNES *et al.*, 2004). A second stage occurs after the sorption process, which is associated with a decrease in sorption due to the gradual exhaustion of the active sites. Later, the desorption of the compound into the aqueous phase may occur (BANIHASHEMI; DROSTE, 2014).

The $\log K_{ow}$ is frequently associated with the sorption of emerging contaminants into the sludge. It has been accepted that compounds with $\log K_{ow} > 4,0$ tend to present high sorption potential, while compounds exhibiting $\log K_{ow} < 2,5$ usually present lower sorption ability (ROGERS, 1996). For E2, EE2 and BPA which are a non-polar, with K_{ow}

equal of 3.94, 3.67 and 3.30, respectively, adsorption might represent an important removal route.

The acidity determined by the functional group of a compound is an important factor in chemical or electrostatic adsorption. Schäfer *et al.* (2011) indicated that, at pH values lower than the pKa, the phenolic hydroxyl groups of the hormones dissociate, becoming negatively charged, making it difficult for the sludge to attract the compounds, and, consequently, be removed by sorption. On the other hand, for compounds with $K_d < 300 \text{ L kg}^{-1}$, sorption can be considered insignificant (LUO *et al.*, 2014).

The biodegradability of a compound is also dependent on its complexity and functional groups. In general, readily biodegradable substances include linear short-chain compounds, unsaturated aliphatic compounds, and compounds with electron-donating functional groups, such as hydroxyl groups or primary amines. In contrast, the removal of hydrophilic compounds containing electron-accepting functional groups is low (below 20%) (GRANDCLÉMENT *et al.*, 2017).

Biodegradation in WWTPs is related to the biochemical reactions induced by the presence of microorganisms, such as bacteria, which assimilate the pollutants. In metabolism, the microorganisms utilize some emerging contaminants as primary substrates for growth or maintenance. The compounds must not be toxic or harmful and must also be present in a high concentration to allow the bacteria to metabolize them and thus obtain energy (TRAN *et al.*, 2013). For the co-metabolism, emerging contaminants are utilized as secondary substrates in conjunction with a primary substrate; the microorganisms do not receive energy or carbon for cell growth from the process (TRAN *et al.*, 2013). It has been reported that the biodegradation of these compounds in WWTPs is favored via co-metabolism because they are usually toxic and often present in trace levels (ng L^{-1} to $\mu\text{g L}^{-1}$) (TRAN *et al.*, 2018).

Biodegradation of emerging contaminants can be achieved by slow-growing bacteria, such as AOB and NOB, which were reported to be important microbial groups responsible for the metabolization of these compounds (MEN *et al.*, 2017; TRAN *et al.*, 2013). However, previous studies observed that heterotrophic bacteria also played a significant role in emerging contaminants biodegradation (MAENG *et al.*, 2013). BPA was mainly removed by heterotrophic bacteria, and a strong negative correlation between BPA removal and nitrification indicated the limited contribution of AOB to BPA biodegradation (ZIELIŃSKA *et al.*, 2014). Furthermore, it was found that AOB and

heterotrophic bacteria could act together for the removal of such contaminants (KHUNJAR *et al.*, 2011; TRAN *et al.*, 2009). Thus, one of the trends in advanced biological wastewater treatment technologies is the combination of different redox conditions to increase the diversity of the microbial community capable of biotransforming a wide range of pollutants, while maintaining a high organic matter and nutrient removal efficiency (LUO *et al.*, 2014; SEMBLANTE *et al.*, 2017). In these systems, different bacteria may cooperate, and their mutual relationship will improve emerging compounds removal. Other important parameters of the biological processes include the biomass concentration that is linked to the microbial activity, as well as the operating conditions, such as hydraulic retention time (HRT) and sludge retention time (SRT) (ALVARINO *et al.*, 2018; PRATUSH *et al.*, 2020).

Most of the emerging contaminants reviewed by GRANDCLÉMENT *et al.* (2017) were preferentially biotransformed under aerobic conditions, such as some pharmaceuticals and hormones. Hormones are easily degraded under aerobic conditions due to the presence of aromatic rings, which are replaced by hydroxyl groups and can be co-metabolized by AOB (CHANG, 1997; PRATUSH *et al.*, 2020). Nitrification generally enhances biotransformation, while anoxic conditions imposed on denitrification units were not especially relevant (GRANDCLÉMENT *et al.*, 2017, SUAREZ *et al.*, 2010).

Autotrophic and heterotrophic biomass were compared in terms of removal of E1, E2, E3, EE2, and BPA (KASSOTAKI *et al.*, 2019). Experiments were performed with enriched nitrifying activated sludge (NAS) and enriched AOB sludge, as well as with conventional activated sludge (CAS). Both enriched NAS and AOB demonstrated a negligible degrading capacity, lower than 14%. Conversely, the biodegradation capability of the heterotrophic fraction of CAS was substantial. E2 and E3 were removed by 100% and 78%, respectively. E1 was found to be the main transformation product of E2, and it was also highly eliminated. Finally, EE2 and BPA were more biologically persistent, with removals ranging from 10% to 39%.

Results are somewhat contradictory, and more research is required to evaluate the contribution of autotrophic and heterotrophic bacteria present in different treatment systems to the removal of EDCs.

2.4 Aerobic granular sludge (AGS)

Aerobic granulation is considered one of the most promising technologies for biological wastewater treatment. Aerobic granules are formed through the self-immobilization of microorganisms composing a high-diversity microbial community, each with a specific function on pollutants degradation (de KREUK; LOOSDRECHT, 2006; WEISSBRODT *et al.*, 2013). Granules show a strong, dense, and regular structure that gives the biomass good settling properties, high retention in the system, and the capacity to withstand load shocks (LIU; TAY, 2004).

The development of aerobic granules was first reported by MISHIMA; NAKAMURA (1991) in a continuous aerobic ascending sludge blanket reactor. Aerobic granules with diameters of 2 to 8 mm and good settling properties were obtained. Since then, aerobic granules have been successfully cultivated by many researchers for the treatment of wastewater containing organic compounds, nitrogen, phosphorus, and diverse toxic substances (BASSIN *et al.* 2012; HE *et al.*, 2017; CYDZIK-KWIATKOWSKA *et al.*, 2017).

2.4.1 Granulation process

Granules are predominantly formed by diverse bacterial communities tightly bound and embedded in a matrix of extracellular polymeric substances (EPS). They comprise polysaccharides (PS), proteins (PN), lipids, nucleic acids, and humus, among other components, where the first two are the major constituents. The content and composition of EPS substantially influence the physicochemical characteristics of microbial aggregates within the granules (RUSANOWSKA *et al.*, 2019). PS is primarily responsible for the stability of the granules, while PN plays a major role in the formation of granular sludge (SARMA *et al.*, 2017).

There are different hypotheses in the literature to explain the granulation process mechanism, but no consensus was reached. Initially, BEUN *et al.* (1999) proposed that the granulation process would start with fungi that would form heavier mycelia than bacteria, being retained in the reactor. These fungal clusters would function as an immobilization matrix in which bacteria could develop into colonies. After a while, due to a lack of oxygen inside, the mycelia break up, but the bacterial colonies can manage to remain in the reactor because they were large enough to settle quickly. These colonies

grow more and more and would form granules. Later it was observed that the presence of fungi was not necessary for granules development.

BARR *et al.* (2010) suggested that there could be two distinct mechanisms in the formation of aerobic granules. Compact, smooth granules could be formed by the gradual growth of a single colony of microorganisms, while relatively loose granules could be formed by the aggregation of many independent colonies of microorganisms. Another hypothesis suggests that the precipitated metal ions, such as Fe^{3+} , are responsible for forming an inorganic substrate to which the microorganisms are coupled to form the granule nucleus. The moderate shear stress ($0.07\text{-}0.2 \text{ L min}^{-1} \text{ L}^{-1}$ of bed) generated by aeration is also essential for the formation of mature granules (TSUNEDA *et al.*, 2003).

Recently, it has been proposed a more detailed granulation mechanism that consists of four steps: (i) cell-to-cell attraction promoted by mechanisms of surface charge neutralization by divalent cations such as Ca^{2+} , preventing the repulsion between the charges, and by van der Waals forces; (ii) microbe-to-microbe attachment by self-connected cells to form aggregates; (iii) microbial adhesion enhancement by secretion of EPS; and (iv) hydrodynamic shear force to stabilize mature granules related to reactor configuration and operating conditions (ZHANG *et al.*, 2016).

Granulation is a complex and organized process affected by numerous operating parameters, including substrate composition and availability, organic loading rate, hydrodynamic shear force, dissolved oxygen (DO), reactor configuration, SRT, settling time, and volume exchange ratio in sequencing batch reactor (SBR) (ADAV *et al.*, 2008). Although all these factors influence the granules properties, only some are associated with the selection of granular particles over flocculent sludge (ROLLEMBERG *et al.*, 2018). The selection pressure is easier achieved in some reactor configurations and operational modes for the successful development and maintenance of granules.

2.4.2 Factors impacting the granulation process

2.4.2.1 Reactor operating conditions

Granules have mainly been cultivated in sequencing batch reactors. The phases – influent feeding, aeration, settling, and treated effluent withdrawal comprise the SBR

cycles that usually last 3 to 6 hours. A short cycle time results in a short HRT, which is positive for rapid granulation (WINKLER *et al.*, 2013).

At the beginning of the cycle, influent containing the pollutants are fed in upflow mode into the reactor, percolating the biomass bed. This phase is usually operated under anaerobic conditions to select for slow-growing organisms such as PAOs. In the sequence, aeration starts. Because of the layers formed in the granule structure, the reaction could happen in aerobic, anoxic, or anaerobic conditions. When the aerated phase ends, a short time of settling, usually between 2 and 10 min, takes place (ADAV *et al.*, 2008), allowing the rapid selection of microbe aggregates, while the flocs with low settling properties are washout from the reactor with the treated effluent. Repeating cycles exerts selection pressure on the composition of the microbial aggregates, allowing the formation of dense, compact, and round-shaped granules (NANCHARAI AH; KUMAR, 2018).

2.3.4.2 Hydrodynamic shear force

Hydrodynamic shear force plays an important role in the structure of the granules, stimulating the secretion of EPS by bacteria, besides affecting the stability and performance of the system, and therefore the rapid formation of compact and dense granules (LIU; TAY, 2002).

The height to diameter ratio ($H D^{-1}$) of a reactor, aeration intensity, and upflow surface air velocity is directly related to the shear force imposed on the system (ADAV *et al.*, 2008). A high ratio of $H D^{-1}$ allows a longer circular trajectory, which generates effective hydraulic attrition of microbial aggregates. Besides, it facilitates an efficient separation between the flocculent and granular sludge during the short settling period (LIU; TAY, 2002).

Aeration has been considered the main parameter to control the shear force. A high aeration rate is favorable for granules stability because it produces a smooth particle surface, avoiding the development of filamentous bacteria (ADAV *et al.*, 2008; HE *et al.*, 2017; LI *et al.*, 2011). HE *et al.* (2019) observed that a moderate aeration intensity favors the system performance under a low carbon nitrogen⁻¹ ($C N^{-1}$) ratio (<4,0).

TAY *et al.* (2001) found that small flocs were dominant when the upflow surface air velocity was 0.3 cm s^{-1} . However, when it was increased to 1.2 cm s^{-1} , denser granules

were obtained. DEVLIN *et al.* (2017) showed that the formation of aerobic granules is possible at low air velocity (0.41 cm s^{-1}), particularly for the treatment of wastewater with relatively low organic matter concentration (Chemical oxygen demand (COD) of 300 mg L^{-1}). However, it was not observed at higher COD (600 or 1200 mg L^{-1}).

2.4.3 Factors impacting the stability of the granules

One of the main challenges in applying AGS technology is the stability of granular biomass for long periods. Usually, reactors are operated for many months and years, during which granules may remain morphologically intact and structurally compact. However, over time, due to an inappropriate operating condition, deterioration of granular properties may occur, and the treatment performance can be impaired. Besides, as described in item 2.4.2, granular biomass stability is affected by substrate composition, organic load, SRT, and other factors (ROLLEMBERG *et al.*, 2018).

2.4.3.1 Substrate composition

Sources of carbon, nutrients, and recalcitrant compounds are present in the influent streams and affect microbial diversity and the spatial distribution of microorganisms. Organic compounds stimulate heterotrophic bacteria growth, while in the presence of organic matter and ammonium, there is a competition for DO and space in the granules between autotrophic and heterotrophic biomass (WANG *et al.*, 2018). Moreover, carbon sources influence AGS performance and EPS secretion (HE *et al.*, 2018).

In previous studies, glucose as a carbon source favored the appearance of filamentous granules, while acetate generated dense and spherical granules (DU *et al.*, 2011; TAY *et al.*, 2002). ROLLEMBERG *et al.* (2019) observed that the granulation process took longer when glucose was used in the influent, and lower microbial diversity was found in the granules. On the other hand, when propionate was used as a carbon source, stable and dense granules were formed, but a longer time was needed to achieve mature granules (LEE *et al.*, 2010; WAN *et al.*, 2015). LONG *et al.* (2015) reported disintegration of granules after a long operating time when acetate was used, a fact related

to the granule size. Therefore, it has been suggested that the mean size of granules should not exceed 2 – 3 mm (WANG *et al.*, 2007).

PRONK *et al.* (2015) observed that the use of methanol as substrate produced methane (CH₄) and carbon dioxide (CO₂) in the anaerobic phase forming unstable and filamentous granules with the predominance of methanogenic organisms at the center of the granules. The use of propionate and acetate in the reactor feeding increased the accumulation of PHA, favoring PAOs and resulting in stable granules.

The substrate also affects the competition between functional groups involved in carbon, nitrogen, and phosphorus removal (PIJUAN *et al.*, 2004). For example, methanol and ethanol are commonly used in many WWTPs to improve denitrification. However, these substrates have a negligible role in biological phosphorus removal. On the other hand, ethanol allowed the formation of stable granules, although they had a lower performance than those cultivated in acetate (ROLLEMBERG *et al.*, 2019). Therefore, VFA as acetate and propionate favor the formation of more stable granules, with acetate as the substrate more easily assimilated by PAOs (LEE *et al.*, 2010).

Concerning C N⁻¹ ratio, high values might cause granules disintegration because of the growth of filamentous microorganisms. KOCATURK; ERGUDER (2016) reported that C N⁻¹ ratios between 10 and 30 favored the appearance of heterotrophic organisms with the predominance of filamentous organisms. On the other hand, low C N⁻¹ ratios, closer to 1.0, caused shifts in the microbial community and reduced the EPS content, impacting the resistance and settling properties of granules (CARRERA *et al.*, 2004; YANG *et al.*, 2005). LUO *et al.* (2014) and PEYONG *et al.* (2012) observed mature granules with a good performance when the C N⁻¹ ratio was between 2 and 10. On other hand, KOCATURK; ERGUDER (2016) observed higher carbon and nitrogen removal at a C N⁻¹ ratio of 7.5.

2.4.3.2 Food to microorganisms ratio (F M⁻¹)

Food to microorganisms ratio (F M⁻¹) has been suggested as a key factor in the aerobic granulation process. WU *et al.* (2018) indicated that a good F M⁻¹ ratio for granular biomass formation is between 0.4 and 0.5 gCOD gVSS⁻¹ d⁻¹, allowing higher microbial diversity and better pollutants removal efficiency. The results corroborate with those found by TAY *et al.* (2004), who reported that 0.33 gCOD gVSS⁻¹ d⁻¹ was enough

for the development of stable granules. KANG; YUAN (2017) observed granules disintegration with a decrease in the $F M^{-1}$ ratio from 0.4 to 0.2 $\text{gCOD gVSS}^{-1} \text{d}^{-1}$. Lower values caused a reduction in EPS production, preventing the granulation process.

The volumetric organic loading rate (ORL) is directly related to the $F M^{-1}$ ratio. A low ORL is related to filamentous organisms and the consequent disintegration of the granules. PEYONG *et al.* (2012) observed this characteristic at ORL of 0.54 $\text{kgCOD m}^{-3} \text{d}^{-1}$. Values between 0.5 and 10 $\text{kgCOD m}^{-3} \text{d}^{-1}$ have been considered as a reference (ROLLEMBERG *et al.*, 2018). LONG *et al.* (2015) and THANH *et al.* (2009) observed mature granules at ORL of 15 $\text{kgCOD m}^{-3} \text{d}^{-1}$. The system instability was attributed to high organic loads because of the increase in granules size, making organic matter penetration more difficult (ZHENG *et al.*, 2006).

2.4.3.3 Salinity

Microbial communities are often incapable of tolerating high osmotic pressures (WANG *et al.*, 2017), which may deteriorate the efficiency of the biological process when the WWTPs receive saline wastewater (LI *et al.*, 2010). Salinity is especially important in coastal cities where seawater is used as an alternative water source for toilet flushing or due to seawater intrusion in sewer networks (WU *et al.*, 2008). Besides, many industrial wastewaters, such as those from fish canning industries, can present high salinity in their composition (GRAAFF *et al.*, 2019).

Recent studies have found that AGS remained stable under relatively low salt concentrations (0.25–1.5% NaCl) (OLIVEIRA *et al.*, 2021; WANG *et al.*, 2017). LI *et al.* (2019) reported a good performance of an AGS-SBR operating at relatively low salinity (1%). However, at 2 and 4% salinity levels (in terms of NaCl e K_2SO_4 , w/w = 1:1), LI *et al.* (2019) noticed a deterioration of organic pollutants removal efficiency. Nitrite oxidation and phosphate removal were also reported to be inhibited at 2% Cl⁻ (PRONK *et al.*, 2014). BASSIN *et al.* (2011) have observed that high salinities (3.3% NaCl) strongly impacted phosphorus removal capability in an AGS system by favoring GAOs over PAOs.

2.4.4 Microbial composition in the aerobic granules

Aerobic granules present in their structure multiple kinds of microbes such as nitrifiers, denitrifiers, PAOs, and GAOs. The granule structure has different layers due to diffusional limitations of the DO and substrate concentration gradients (de KREUK; van LOOSDRECHT, 2006). For this reason, the outermost granule zone is maintained in an aerobic condition, followed by an anoxic and anaerobic region in the inner core (Figure. 2.2). The granule stratification allows simultaneous nitrification, denitrification, and phosphorus removal (SNDPR), so that COD and nutrients can be removed in a single reactor compartment.

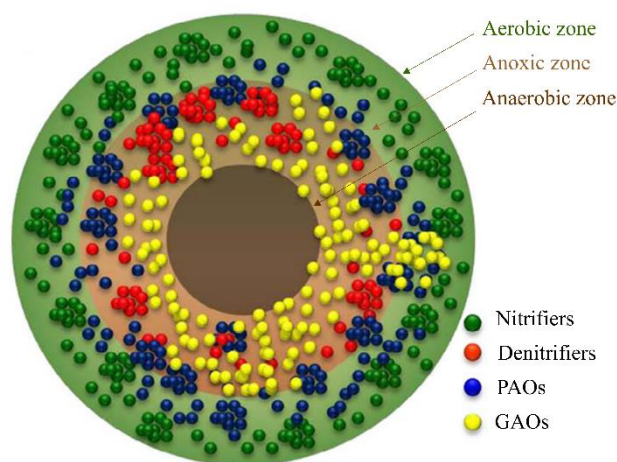


Figure 2.2 – Aerobic granular sludge regions and the microbial community typically found in each layer.

Source: Adapted from Winkler *et al.* (2013).

There is a competition for space and oxygen between heterotrophic and autotrophic microorganisms because of the simultaneous removal of organic matter and ammonium. The heterotrophic microorganisms, characterized by high growth rates, are located in the external layers, while the slow-growing autotrophs are found in deeper layers, where the oxygen availability is limited (van LOOSDRECHT *et al.*, 1995). The latter have shown a positive influence on the stability of the granules (de KREUK; van LOOSDRECHT, 2004; LIU *et al.*, 2004).

Besides the slow-growing nitrifiers, de KREUK; van LOODRECHT (2004) suggested that PAOs and GAOs are also important to the formation and stability of the granules. However, there is competition between these two groups because both consume the same substrate, VFA, and present similar metabolism. Nevertheless, GAOs only

compete with PAOs for the available substrate and do not accumulate phosphate, which may deteriorate the phosphorus removal efficiency (BASSIN *et al.*, 2012).

Carta *et al.* (2001) showed that the DPAOs and denitrifying glycogen accumulating organisms (DGAOs) are also considered slow-growing organisms. These organisms consume polymers internally stored inside the bacteria, such as PHA, as an electron donor to reduce nitrite and nitrate. In the case of DPAOs, they also contribute to anoxic phosphate removal.

The selection of the desired microorganisms is required for good performance of the AGS reactor (ROLLEMBERG *et al.*, 2018). The main strategy consists of an anaerobic feeding period followed by an aerobic (selection of PAOs and GAOs) or anoxic phase (selection of DPAOs and DGAOs) (CARTA *et al.*, 2001). The application of a regime alternating the presence (feast) and absence (famine) of substrate decreases the growth rate of heterotrophic organisms, particularly during the famine phase. In the anaerobic period, the easily biodegradable substrate is absorbed and converted into intracellular polymers (PHA), and their utilization by slow-growing bacteria under aerobic or anoxic conditions is essential to achieve stable granulation (PRONK *et al.*, 2015).

2.4.5 Organic matter and nutrients removal

Different processes occur within the granules due to the feast and famine periods throughout of operating cycle. A slow, ascending filling regime under anaerobic conditions is used to guarantee enough contact time between the biomass and the influent and to promote the growth of PAOs. In this condition, the feeding acts as a feast period, during which the external carbon concentration is high. The substrate diffuses into the granules and is stored as intracellular polymers, such as PHA. In this phase, denitrification of the NO_x^- compounds remaining from the previous cycle may occur using the available external source of carbon (BASSIN, 2011).

The following aerobic phase acts as a famine period since there is little or no available external carbon source. Only the stored substrate inside the cells, in the form of PHA, is available for the microorganisms. The PAOs use the stored substrate for maintenance and growth, the latter occurring at a lower rate than those presented by heterotrophic organisms that consume the organic matter in the presence of external

electron acceptors (oxygen and nitrite/nitrate). Moreover, during the aerated period, PAOs take up the phosphate, resulting in the effective removal of phosphorus from the bulk liquid. The absorbed phosphate is removed from the system with the sludge withdrawal. The nitrification products (nitrite and nitrate) can be reduced to nitrogen gas through denitrification inside the granules, where anoxic conditions can be established, using the stored substrate as a carbon source (LEMAIRE *et al.*, 2008). Figure 2.3 shows the main reactions that occur inside an aerobic granule.

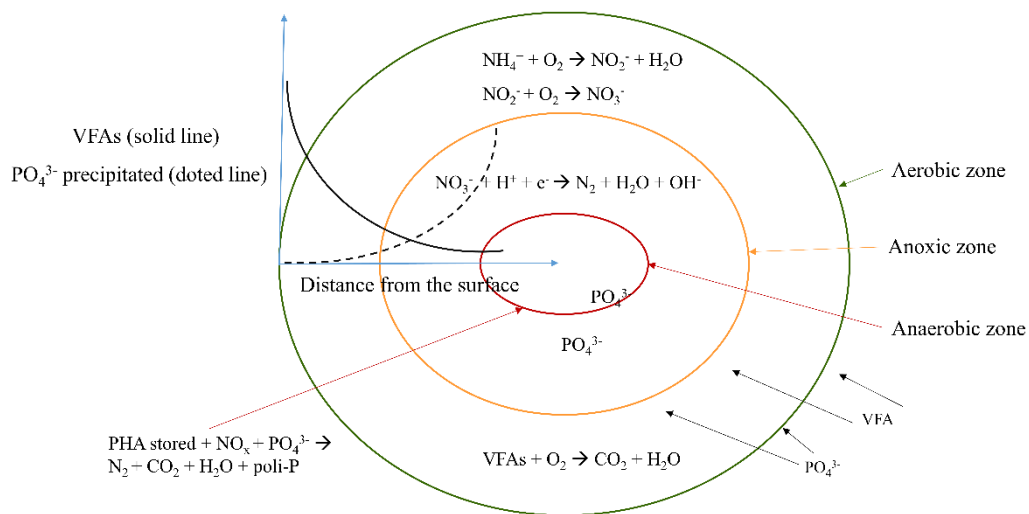


Figure 2.3 - Transformations that occur in each layer of a stratified aerobic granule.

Source: Adapted from SARMA; TAY (2018).

The correct operation of a reactor is essential for achieving high combined organic matter and nutrients. It is appropriate that most, if not all, organic matter is consumed under anaerobic conditions because this conversion directly influences PAOs selection over other heterotrophic organisms. In this way, the anaerobic feeding must be long enough to allow the complete consumption of the substrate by PAOs (BASSIN, 2018).

2.4.5.1 Nitrogen removal

Aerobic and anoxic conditions are required to achieve biological nitrogen removal. As shown in Figure 2.3, the structure of aerobic granules provides the presence of different redox conditions, with aerobic and anoxic zones. Microorganisms responsible for nitrification are found in the aerobic zone, whereas in the anoxic zone, heterotrophic

denitrifying bacteria are present (GAO *et al.*, 2011). As a result of this layout, it is usually claimed that AGS technology allows simultaneous nitrification-denitrification (SND) within the same reactor, even under aerated conditions (NANCHARAI AH *et al.*, 2016).

Although nearly complete ammonium removal can be achieved, total nitrogen removal by AGS is still challenging. Nitrate is one of the major contributors to total nitrogen in the effluent. The success of heterotrophic denitrification processes depends on the VFA concentration in the wastewater. Denitrifying bacteria convert the nitrification products (nitrite and nitrate) using intracellular PHA stored as a carbon source. Denitrification efficiency by AGS is dependent on the granule diameter (De KREUK *et al.*, 2005) due to the diffusion of oxygen inside the granular particles. As the diameter of the granule decreases, more oxygen can penetrate the anoxic zone. Moreover, the concentration of available substrate is expected to decrease. This situation is detrimental to denitrification, and it may limit the ability of AGS to achieve total nitrogen removal.

For enhancing biological nitrogen removal, another strategy is to introduce an anoxic phase after the aerobic phase, alternating the nitrification and denitrification processes (ADAV *et al.*, 2009). The non-availability of COD for denitrification and P release in the anoxic phase could be challenging for the system (LOCHMATTER *et al.*, 2014). The addition of VFA to the wastewater can be interesting (TONG; CHEN, 2007). For this purpose, the fermentation of primary sludge could produce VFA in WWTPs (BOUZAS *et al.*, 2007). However, as the diameter of the granule increases, denitrification efficiency could be limited by poor mass transfer and low VFA concentration in the anoxic zones (SARMA; TAY, 2018).

2.4.5.2 Phosphorus removal

Phosphorus removal in AGS can be achieved by biological and physicochemical processes: EBPR, precipitation within the granules, and accumulation by EPS.

The biological process involves the accumulation of phosphorus by PAOs as intracellular polyphosphate, which is stored in their cells to generate energy. The generated energy is used to convert biodegradable organic matter, such as VFA, mainly as intracellular PHAs (Van LOOSDRECHT *et al.*, 1997). When the condition changes to aerobic, in the absence of enough amount of organic carbon, stored PHA is utilized as

energy for phosphorus uptake that is accumulated within the cell as polyphosphates (ZHANG *et al.*, 2013).

The second mechanism involved in phosphorus removal by AGS is precipitation. In wastewater, phosphorus is mainly found as soluble phosphate. Since the wastewater has a high concentration of VFA (externally added), the cations would be mostly found as soluble cation–VFA complexes. Oxidation of VFA and their low concentration would allow the cations (Ca^{2+} , Mg^{2+} , Fe^{3+}) and the soluble phosphate to form new complexes (cation–phosphate), which will precipitate (SARMA; TAY, 2018). The denitrification process collaborates with the phosphorus precipitation because the denitrifying bacteria consume the VFA present around the core region, causing the pH to increase. This would force the soluble phosphorus to precipitate within this region (SARMA; TAY, 2018).

Phosphorus accumulation by EPS is also considered a phosphorus removal mechanism. The presence of multivalent cations in the EPS matrix, such as K^+ , Mg^{2+} , and Ca^{2+} , was reported to affect phosphorus uptake (WANG *et al.*, 2014). SARMA; TAY (2018) hypothesized that the presence of an excess of multivalent cations, but limited amounts of chelating agents (e.g., VFA), modifies ionic bridges present in EPS matrix and accommodates phosphate groups. However, if both PO_4^{3-} groups and free cations concentration increase, it would allow the precipitation of minerals like $\text{Ca}_3(\text{PO}_4)_2$ (calcium phosphate), $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ (hydroxy-apatite), and others.

2.5 Aerobic granular sludge for the treatment of emerging contaminants

Some researchers have studied the biodegradation of several emerging contaminants in aerobic granular sludge systems. MARGOT *et al.* (2016) studied the removal of 36 micropollutants at $1 \mu\text{g L}^{-1}$ from synthetic wastewater. The removal of nine of these compounds was over 80%, but for 17 compounds, the removal percentage was less than 20%. Five substances, including BPA, were better removed in the reactor with nitrification, probably due to co-oxidation catalyzed by the enzyme ammonium monooxygenase (AMO). Thus, it is likely that both AOB and heterotrophic organisms were responsible for BPA removal. However, for the other micropollutants, AOB did not seem to play a significant role in their removal. Many compounds were better removed under aerobic conditions, suggesting that heterotrophic microorganisms were involved in their degradation.

WANG; IVANOV (2009) studied the biodegradation of estrogens E1, E2, E3, and EE2 (100 $\mu\text{g L}^{-1}$ of each) by microbial granules containing nitrifying bacteria. The system reached the steady-state in terms of carbon removal and complete nitrification after 40 days. Nitrite was not detected in the effluent, and the conversion of ammonium to nitrate was 93%. The complete biodegradation of the mixture of 100 $\mu\text{g L}^{-1}$ of each compound occurred in 60 days. Furthermore, the authors found that the adsorption of E1 and EE2 on granules was 407 ng g^{-1} and 83 ng g^{-1} , respectively. However, the estrogenic fraction found in the granules represented less than 0.5% of the total concentration (E1 + EE2) in the batch experiments. This means that biodegradation was the major process in estrogens reduction. In contrast, ZHENG *et al.* (2015) showed that AGS high potential of adsorbing E2. When the initial concentration of E2 was 400 $\mu\text{g L}^{-1}$, the equilibrium concentration in the granules was 141.4 $\mu\text{g g}^{-1}$. The authors also observed that the adsorption rate decreased with increasing E2 concentration. External diffusion of the compound depends mainly on the solute concentration, the agitation speed and the external surface area of the granules, which is affected by the size, shape and density of the particles.

CASTELLANOS *et al.* (2021) obtained the removal of about 99% for E2 and 93% for EE2 in an AGS-SBR, both present in the influent at a concentration of 20 $\mu\text{g L}^{-1}$. E2 and EE2 profiles over the SBR cycle suggested rapid initial adsorption on the granular biomass occurring anaerobically, followed by biodegradation under aeration. Through yeast estrogen screen assays, an average moderate residual estrogenic activity (0.09 $\mu\text{g L}^{-1}$ EQ-E2) was found in the samples analyzed. Ammonium removal dropped from 97% to 58% when the reactor was fed with estrogens. However, gradual recovery of nitrifying activity was observed. Phosphate removal also became unstable in the presence of estrogens, decreasing from 100% to 67%. Nevertheless, these compounds seemed to have only an initial adverse effect on AOB and PAOs, because the complete ammonium and phosphate removal was recovered (CASTELLANOS *et al.*, 2021).

WANG *et al.* (2021) studied the effect of nitrifying sludge granulation on EE2 removal. Around 35% removal was achieved from the influent wastewater containing 500 $\mu\text{g L}^{-1}$ of this compound. The dense structure of granular sludge resulted in nitrite accumulation and this might be a key factor affecting EE2 removal. The physical structure of nitrifying granules could reduce the negative effects of EE2 on AOB, which could degrade EE2 via co-metabolism.

Addressing E1 biodegradation by AGS, PETERSON *et al.* (2017) found stable granules, with nitrogen removal of 61 to 87% and COD removal higher than 90%. However, the system provided a limited removal of E1, with an average effluent concentration of $10.4 \pm 3.9 \mu\text{g L}^{-1}$, close to that of influent. The authors stated that the high influent COD selected fast-growing organisms instead of slow-growing bacteria capable of degrading E1. When the influent COD was reduced (1000 to 200 mg L^{-1}), carbon was effectively degraded, and E1 removal significantly improved (effluent concentration $\leq 0.82 \mu\text{g L}^{-1}$). However, granulation did not occur, negatively affecting nitrogen removal. Thus, it is likely that lower COD may allow the growth of E1-degrading microorganisms, despite the loss of nitrogen removal capacity.

Using a sequencing batch biofilter granular reactor, with a high SRT (up to 6 months) and high biomass concentration (up to 40 g L^{-1}), BALEST *et al.* (2008) investigated the removal efficiency of E1, E2, BPA and 4-tert-octylphenol (4t-OP)) from municipal wastewater. The average removal obtained during the 4-months operating period were 60%, 69%, 93%, and 81% for compounds E1, E2, BPA, and 4t-OP, respectively. The studied system also showed good COD and ammonium removal efficiencies, both around 90%.

The removal of EE2, together with 4-nonylphenol and carbamazepine at a concentration of 500 $\mu\text{g L}^{-1}$, was assessed in an AGS-SBR (KENT; TAY, 2019). In general, the main removal mechanism for the emerging contaminants was initially adsorption but it decreased when active sites were saturated, while biodegradation improved as biomass acclimated. The stable degradation for EE2 was 16.09 $\mu\text{g g}^{-1}$. The performance of the reactor was affected by the presence of the compounds; however, high N and P removal efficiency was maintained.

In addition to estrogens, other emerging compounds have also been studied in AGS systems. AMORIM *et al.* (2016) fed an AGS-SBR with synthetic effluent containing a mixture of chiral drugs (CPs), each at 1.3 $\mu\text{g L}^{-1}$. Sorption seemed to be the main mechanism for CPs removal. A gradual decline in CPs removal was observed, probably related to the decrease in the adsorption capacity of the granules. It was observed that COD after anaerobic feeding increased, and P-release decreased, probably because PAOs activity was affected. Both stages of nitrification were also affected. However, the oxidation of nitrite to nitrate was more affected. Granule morphology was temporarily affected by exposure to CPs, which led to a decrease in granule size accompanied by an

increase in biomass washing and deterioration of sludge sedimentation properties. After stopping CPs feeding, the biomass started to recover its compact structure, and the system returned to its normal performance concerning COD and phosphate removal. However, the microbial community has not returned to its original composition. Fluctuations in the abundance of PAOs, AOB, and NOB occurred, but these communities likely persisted within the granules during CPs exposure, preventing total system failure and allowing rapid and almost complete recovery of the biological removal processes (AMORIM *et al.*, 2018).

KANG *et al.* (2018) investigated the removal of the antibiotic sulfamethoxazole (SMX) at a concentration of $2 \mu\text{g L}^{-1}$ in an AGS reactor. SMX removal efficiency was 84%, and the presence of the compound had no significant impact on treatment performance. During the aerobic phase, the elimination of SMX was obtained when COD and ammonium were removed, while sorption and the anoxic/anaerobic phase had no relevant role in this conversion. However, exposure to $2 \mu\text{g L}^{-1}$ of SMX altered the composition of the microbial community after two months of operation. *Rhodocyclaceae*, *Zoogloea*, *Shewanella*, and *Aeromonas* were found to have SMX resistance genes because the relative abundance increased over the operating time (KANG *et al.*, 2018b).

Another toxic compound, 2-fluorophenol (2-FP), at a concentration of $24 \mu\text{g L}^{-1}$, was fed into an AGS-SBR in a study carried out by DUQUE *et al.* (2011). For three months, no biodegradation of the compound was observed. After this period, bioaugmentation with a specialized bacterial strain able to degrade 2-FP was subsequently performed. Total degradation of the compound was achieved with fluoride release, even when the concentration of this compound was doubled. The 2-FP degrading strain was successfully retained by aerobic granules.

Some authors have also studied the effect of emerging contaminants on AGS-based systems at higher concentrations, in the order of mg L^{-1} . CYDZIK-KWIATKOWSKA *et al.* (2017) evaluated BPA concentrations from 0 to 12 mg L^{-1} . BPA removal efficiency with 8 h-cycles exceeded 97% at 6 mg L^{-1} concentration. Ammonium removal started after the BPA concentration dropped to less than 2 mg L^{-1} . As the BPA load was increased, the production of biomass and EPS decreased, and the diameter of the granules increased. Thus, the authors suggested that the granule diameter affected the distribution of AOB and NOB in the granules, and the microbiota in the granules included not only aerobic bacterial genera such as *Aquimonas* and *Pseudoxanthomonas*, but also

anoxic and anaerobic bacterial genera, including *Thauera* and *Azoarcus*. Abundances of *Methylobacillus* sp., *Nitrosospira* sp. and *Sphingomonas* sp. suggested that BPA was both co-metabolized and directly biodegraded. No metabolites were formed, which can be explained by the microbial composition of the granules and the cooperation of microorganisms. LI *et al.* (2015) found that BPA inhibited the activity of heterotrophic and nitrifying bacteria at a concentration of 40 mg L⁻¹. The authors found that the EPS production increased, mainly caused by the increase in the concentration of protein, which may be related to the toxicity of BPA.

AMORIM *et al.* (2014) also studied the effect of fluoroquinolones (FQs), namely ofloxacin, norfloxacin, and ciprofloxacin, at concentrations of 3 to 12 mg L⁻¹, in the operation of an AGS system. The biodegradation of the compounds was not observed, but they were adsorbed on the granules, being gradually released into the bulk liquid in successive cycles after stopping the FQ feeding. AOB and NOB activity was not affected. However, denitrification was impaired, probably due to the decrease in granule diameter. In addition, PAOs were affected, as indicated by a decrease in both P-release under anaerobic conditions and P-absorption in the aerated period. The microbial community predominantly belonged to the α - and γ -branch of the Proteobacteria phylum. The system was capable of returning to its initial conditions after FQ removal. On the other hand, when MOREIRA *et al.* (2015) added fluoxetine at concentrations around 1.2 mg L⁻¹, there was an initial decrease in the efficiency of ammonium and phosphorus removal, but a gradual adaptation was observed for both nitrifiers and PAOs, with a total recovery after 20 and 70 days for N and P-removal, respectively. A shift in the microbial community occurred and probably contributed to the adaption to the fluoxetine load.

3 UNRAVELLING THE REMOVAL MECHANISMS OF ENDOCRINE DISRUPTING COMPOUNDS BY AEROBIC GRANULES

3.1 Introduction

Endocrine-disrupting compounds (EDCs) are a relevant group of emerging contaminants due to their worldwide detection and the potential adverse effects on living organisms (CABALLERO-GALLARDO *et al.*, 2016; CARNEVALI *et al.*, 2018). EDCs include many active substances, such as natural and synthetic hormones and xenoestrogens. The extensive use of these compounds makes their release into the environment inevitable (TING; PRAVEENA, 2017). Most EDCs reach the aquatic environment through domestic or industrial wastewaters due to the inefficiency of wastewater treatment plants (WWTP). Furthermore, many countries, especially developing ones, release their wastewater in natura into the water bodies, with the compounds being excreted in non-metabolized forms (LUO *et al.*, 2014).

17 β -estradiol (E2), bisphenol-A (BPA), and 17 α -ethinylestradiol (EE2) are important EDCs detected in surface and potable waters in many countries from North America and Europe (ALVARINO *et al.*, 2018; TRAN *et al.*, 2018) and Asia, mainly China (BEN *et al.*, 2018; MOREIRA *et al.*, 2021). In Brazil, Ghiselli (2006) reported that similar concentrations were found in raw and treated domestic wastewater samples, indicating that these compounds were not effectively removed in WWTPs. In a study comprising 10 years of analyses of different samples (raw and treated sewage, surface, ground and drinking waters), carried out in the state of São Paulo (Brazil), MONTAGNER *et al.* (2019) identified potential risks for aquatic life posed by EE2, E2, and BPA, among others emerging contaminants in a preliminary risk assessment. However, for the drinking water criteria adopted, only E2 showed adverse effects on human health at the concentrations found in the studied scenario (MONTAGNER *et al.*, 2019).

E2 is a natural sex hormone produced by the ovarian follicles, being biotransformed in the liver to estrone (E1) and estriol (E3). The latter is the main by-product eliminated in urine and feces (BRUNTON *et al.*, 2005). On the other hand, EE2

is a synthetic estrogen derived from E2 and used in almost all formulations of oral contraceptive pills and medications for the menopausal and postmenopausal syndrome, treatment of prostatic and breast cancer, and others (ARIS *et al.*, 2014). BPA is produced worldwide and used to manufacture polycarbonate plastic and epoxy resin used to pack most food and beverage cans (CORRE *et al.*, 2015). This compound is commonly released into the environment through the industrial effluents generated by industries that use BPA in their manufacturing or processing facilities (ZIELIŃSKA *et al.*, 2014).

Conventional WWTPs are designed to remove organic matter, nutrients and pathogens, but not specifically to eliminate emerging contaminants (SANSON, 2012; TRAN *et al.*, 2018). The presence of these substances may inhibit or affect the microbial community in the biologic treatment, compromising its performance (BOSHIR *et al.*, 2017; GARCIA-BECERRA; ORTIZ, 2018). Aerobic granular sludge (AGS) emerged as a promising technology for wastewater biological treatment, especially in the presence of toxic substances (ADAV *et al.*, 2008; ROLLEMBERG *et al.*, 2018). Aerobic granules are characterized by a dense and regular structure, allowing good settling properties and high biomass retention to be achieved (LIU; TAY, 2004). Moreover, they are composed of a highly diverse microbial community with multiple functions in biological treatment. The interrelationship of the different bacterial functional groups present in AGS may enhance process robustness against inhibition and therefore improve the degradation of pollutants that are difficult to biodegrade (KREUK; LOOSDRECHT, 2006; WEISSBRODT *et al.*, 2013).

The emerging contaminants can be removed in a biological reactor through biotransformation, sorption, and volatilization (often with an insignificant role in overall removal) (ALVARINO *et al.*, 2018). Most studies on the removal of emerging contaminants by AGS focused on evaluating their effect on process performance and microbial community (CYDZIK-KWIATKOWSKA *et al.*, 2017; CASTELLANOS *et al.*, 2021a; WANG *et al.*, 2021), but only a few studies distinguished the role of adsorption and biodegradation in EDCs removal. KENT; TAY (2019) reported adsorption as the main removal mechanism of EE2. However, its role became less important as active sites became saturated, while biodegradation was enhanced upon biomass acclimation. A similar result was obtained by CASTELLANOS *et al.* (2021a). In their study, the profiles of E2 and EE2 over the AGS-sequencing batch reactor (SBR) cycle suggested rapid initial adsorption on the granular biomass occurring anaerobically,

followed by biodegradation under aeration. However, none of the previous studies addressed the amount of EDCs sorbed on the granular biomass in each phase of an SBR cycle, either under anaerobic or aerobic conditions. Such information is important to evaluate the contribution of different mechanisms and pathways to the overall removal attained in the bioreactor under distinct redox conditions.

Therefore, this study aimed to evaluate: 1) the capability of AGS to remove E2, BPA, and EE2 from simulated domestic wastewater; 2) how these compounds impact the organic matter, nitrogen, and phosphate removal conversions and the physical properties of aerobic granules; and 3) the contribution of adsorption and biodegradation for the removal of the target compounds in different phases of an SBR cycle operated under alternating anaerobic-aerobic conditions. Dynamic changes of bacterial community properties (diversity, richness and composition) under exposure to EDCs mixture were also assessed by 16S rRNA gene sequencing, and the results were associated with reactor performance.

3.2 Materials and methods

3.2.1 Chemicals

E2, EE2, and BPA standards were purchased from Sigma Aldrich with a purity degree above 98% (Steinheim, Germany). HPLC grade methanol and acetonitrile were purchased from Tedia (Brazil). Ultrapure water was supplied by a Milli-Q water system. Stock solutions of the EDCs mixtures were prepared every two or three days at a concentration of 20 g L⁻¹ in methanol and stored under refrigeration in amber glass bottles. The reactor influent synthetic wastewater (de KREUK *et al.*, 2005) was prepared with analytical-grade chemicals (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

3.2.2. AGS reactor configuration

The AGS system was operated discontinuously as an SBR and consisted of a lab-scale cylindrical plexiglass column, with 79 cm of useful height, 5 cm of internal diameter, and 1.5 L of working volume. A programmable logic controller (PLC) was used

to start/stop pumps and open/close valves for influent feeding, aeration, and effluent withdrawal. Each cycle lasted 3 h (180 min) and consisted of four consecutive phases: filling (60 min), during which 0.95 L of influent wastewater was pumped into the bioreactor under anaerobic conditions; aeration (112 min), during which compressed air was sparged into the reactor bottom at a flow of 4 L min⁻¹; biomass settling (3 min) and effluent withdrawal (5 min). The anaerobic-aerobic profile of the SBR cycle was chosen to allow simultaneous nitrogen and phosphate removal. The volume exchange ratio of each SBR cycle was 63% and, therefore, the hydraulic retention time (HRT) was around 4.7 h. The sludge retention time (SRT) was controlled between 15 and 25 days by manual excess biomass discharge and natural suspended solids washout during the effluent withdrawal phase. pH values and dissolved oxygen (DO) concentrations were not controlled but remained approximately constant at 7.5 ± 0.5 and 6 ± 1 mg L⁻¹, respectively. The bioreactor was operated at room temperature (25 ± 2 °C). The reactor was inoculated with granular sludge from the same lab-scale reactor operating under similar conditions.

3.2.3 Wastewater composition and reactor operating conditions

The SBR influent wastewater was composed of two different solutions (A and B), as described by BASSIN *et al.* (2012). Solution A comprised the carbon source (provided as acetate) along with other compounds: NaCH₃COO 3H₂O (38.1 mM), MgSO₄·7H₂O (1.29 mM), KCl (4.8 mM), and CaCl₂·2H₂O (0.41 mM). Solution B provided the nutrients (N and P) sources: NH₄Cl (21.4 mM), K₂HPO₄ (2.1 mM), KH₂PO₄ (1.1 mM). A trace element solution (VISHNIAC; SANTER, 1957) was added to the latter at a proportion of 5 mL/L of wastewater prepared to favor the growth of microorganisms. A volume of 150 mL of solutions A and B was mixed with 650 mL of tap water before being fed to the reactor to obtain the desired composition, typically found in domestic wastewater: chemical oxygen demand (COD) of 400 mg L⁻¹, ammonium of 50 mgN L⁻¹ and phosphate of 15 mgP L⁻¹. A third solution (Solution C) was prepared with the EDCs mixture. For this purpose, an appropriate volume of the stock solution containing the target compounds (20 g L⁻¹ of E2, EE2 and BPA) was diluted with tap water in another container consisting of a dark glass bottle to avoid photodegradation of micropollutants. Thus, a volume of 150 mL of each solutions (A, B and C) was mixed with 500 mL of tap water to reach an

influent concentration of about 1 mg L⁻¹ of each target compound. This concentration, despite being higher than those commonly found in the environment, was used to better understand the adsorption and biodegradation mechanisms.

The bioreactor operation was divided into five experimental phases (Table 3.1). Phase I corresponded to the start of reactor operation. At that time, free-EDCs synthetic wastewater was fed to the reactor until the system reached steady-state conditions (i.e., C, N and P conversions stable over time). In phase II, the SBR was fed with synthetic wastewater amended with a mixture of E2 and BPA. During phase III, EE2 was also added to the influent stream. The latter was subsequently removed so that only E2 and BPA were kept in the wastewater (phase IV). The EE2 was added later because this compound is considered less biodegradable, so it could cause adverse effects on the system performance. In the last run (phase V), the reactor returned to the initial imposed conditions (EDCs-free condition).

Table 3.1 - Summary of the operating conditions tested in the SBR.

Phase	Days	EDCs
I	1 – 30	Without EDCs
II	31 – 44	E2 and BPA
III	45 – 101	E2, BPA and EE2
IV	102 – 117	E2 and BPA
V	118 – 128	Without EDCs

3.2.4 EDCs quantification

3.2.4.1 Quantification in the liquid phase

Liquid samples were regularly collected at the bioreactor influent, after the anaerobic feeding phase, and effluent. They were filtered through a 0.45 µm cellulose nitrate membrane to remove any suspended solids that might be present.

The mass of each compound over the AGS-SBR cycle, i.e., for the influent (M_{inf} in mg), after the anaerobic feeding (M_{anaer} in mg), and effluent (M_{eff} in mg), was calculated by Equations (3.1), (3.2) and (3.3), respectively.

$$M_{inf} = C_{inf} \times V_{inf} \quad (3.1)$$

$$M_{\text{anaer}} = C_{\text{anaer}} \times V_T \quad (3.2)$$

$$M_{\text{eff}} = C_{\text{eff}} \times V_{\text{eff}} \quad (3.3)$$

Where C_{inf} , C_{anaer} and C_{eff} represent the average concentration (mg L^{-1}) of each EDC in the influent, after the anaerobic feeding and effluent, respectively, V_{inf} and V_{eff} are the volume of influent wastewater fed to the reactor and treated effluent discharged per cycle (0.95 L), and V_T corresponds to useful reactor volume (1.5 L).

To calculate the removal efficiency in each phase of the SBR cycle and the overall removal (all expressed in percentage), Equations (3.4), (3.5) and (3.6) were used:

$$\text{rem}_{\text{anaer}} = \frac{(C_{\text{inf}} \times V_{\text{inf}}) + (C_{\text{eff}_{n-1}} \times V_{\text{rem}}) - (C_{\text{anaer}} \times V_T)}{(C_{\text{inf}} \times V_{\text{inf}}) + (C_{\text{eff}_{n-1}} \times V_{\text{rem}})} \times 100 \quad (3.4)$$

$$\text{rem}_{\text{aer}} = \frac{(C_{\text{anaer}} \times V_T) - (C_{\text{eff}} \times V_{\text{eff}})}{(C_{\text{inf}} \times V_{\text{inf}}) + (C_{\text{eff}_{n-1}} \times V_{\text{rem}})} \times 100 \quad (3.5)$$

$$\text{rem}_T = \frac{(C_{\text{inf}} \times V_{\text{inf}}) + (C_{\text{eff}_{n-1}} \times V_{\text{rem}}) - (C_{\text{eff}} \times V_{\text{eff}})}{(C_{\text{inf}} \times V_{\text{inf}}) + (C_{\text{eff}_{n-1}} \times V_{\text{rem}})} \times 100 \quad (3.6)$$

Where $C_{\text{eff}_{n-1}}$ represents the effluent concentration from the previous cycle and V_{rem} is the remaining liquid volume at the end of the cycle (0.55 L).

3.2.4.2 Extraction of the EDCs from the granular biomass

For the quantification of EDCs adsorbed on the granular biomass, a methodology based on compound extraction from sediment by TERNES *et al.* (2002) was employed, with some modifications. A homogeneous sample of about 5 g of wet granules collected from the reactor immediately after the anaerobic phase and after the aerated phase was used for the EDCs extraction procedure. A volume of 5 mL of methanol was added together with the sludge sample in a 50 mL falcon tube, under ultrasonic agitation for 10 min. The supernatant was collected and filtered. The procedure was repeated two more times, totalizing 15 mL of liquid at the end of the extraction protocol.

To obtain the mass adsorbed on the total biomass within the SBR, the concentration of the compound (C_{liq}) in the 15 mL liquid sample (V_{analysed}) obtained from 5 g of wet sludge sample after anaerobic feeding or effluent ($M_{\text{s_sample}}$) was extrapolated to the total mass of sludge present inside the reactor (M_{s}). This was obtained considering the volume of the sludge bed (V_{bed}) and the density of the wet granules (ρ) (the average of $1,000 \text{ g L}^{-1}$ was considered) using Equation (3.7).

$$M_S = \frac{C_{\text{liq}} \times V_{\text{analysed}} \times V_{\text{bed}} \times \rho}{M_{\text{s_sample}}} \quad (3.7)$$

To differentiate between the amount of EDCs removed by adsorption and biodegradation in each phase of the SBR cycle, some calculations were performed (Equations 3.8 – 3.21, given in Table 3.2). With the theoretical values of the EDCs in the bulk liquid and the sludge, estimated from the mass of each compound expected to be detected if there was no removal, it was possible to calculate the mass that somehow was removed during the cycle in each phase. Thus, the contribution of adsorption and biodegradation for the removal of target compounds could be assessed.

Table 3.2 - Equations used to estimate the EDCs removal percentage by adsorption and biodegradation in each period of the SBR cycle (anaerobic feeding and aerated period).

	Anaerobic feeding		Aerated period	
Expected mass in the bulk liquid (mg)	$M_{bl_exp_anaer} = (C_{inf} \times V_{inf}) + (C_{eff_n-1} \times V_{rem})$	(3.8)	$M_{bl_exp_aer} = C_{anaer} \times V_T$	(3.9)
Expected mass in the sludge (mg)	$M_{s_exp_anaer} = M_{s_n-1}$	(3.10)	$M_{s_exp_aer} = M_{s_meas_anaer}$	(3.11)
Mass variation	$\Delta_{anaer} = M_{bl_exp_anaer} - M_{bl_meas_anaer}$	(3.12)	$\Delta_{aer} = M_{bl_exp_aer} - M_{bl_meas_aer}$	(3.13)
Removed mass by adsorption (mg)	$M_{removed_ads} = M_{s_n-1} - M_{s_meas_anaer}$	(3.14)	$M_{removed_ads} = M_{s_meas_anaer} - M_{s_meas_aer}$	(3.15)
Total removed mass (mg)	$M_{removed_T} = (C_{inf} \times V_{inf}) + (C_{eff_n-1} \times V_{rem}) - (C_{anaer} \times V_T)$	(3.16)	$M_{removed_T} = (C_{anaer} \times V_T) - (C_{eff} \times V_{eff})$	(3.17)
Adsorption contribution (%)	$CT_{ads} = \frac{M_{removed_ads}}{\Delta_{anaer}} \times 100$	(3.18)	$CT_{ads} = \frac{M_{removed_ads}}{\Delta_{aer}} \times 100$	(3.19)
Biodegradation contribution (%)	$CT_{biod} = \frac{\Delta_{anaer} - M_{removed_ads}}{\Delta_{anaer}} \times 100$	(3.20)	$CT_{biod} = \frac{\Delta_{aer} - M_{removed_ads}}{\Delta_{aer}} \times 100$	(3.21)

Where,

C = concentration;

M = mass;

V = volume;

CT = contribution.

For each letter, there are different subscripted letters, which means:

bl = bulk liquid;

s = sludge;

exp = expected;

meas = measured;

anaer = anaerobic phase;

aer = aerated phase;

inf = influent;

eff = effluent;

rem = remaining;

T = total;

s_{n-1} = sludge from the previous cycle

eff_{n-1} = effluent from the previous cycle;

ads = adsorption;

biod = biodegradation.

To calculate the expected mass in the bulk liquid after anaerobic feeding ($M_{bl_exp_anaer}$) is needed to consider the dilution factor and the concentration remaining from the previous cycle (volume of liquid remaining inside the reactor - 0.55 L) (3.8). The expected mass sorbed in the sludge after anaerobic feeding ($M_{s_exp_anaer}$ - 3.10) is the same that the sorbed mass in the previous cycle (3.9), because the sludge remains inside the reactor from one cycle to another. The variation mass (Δ_{anaer}) is obtained by the difference in mass from the bulk liquid expected to the mass from bulk liquid measured (3.12). The mass that was removed by adsorption ($M_{removed_ads}$) is obtained by the difference in mass from sludge of the end of the previous cycle to the sludge measured after the anaerobic feeding (3.14). The total removed ($M_{removed_T}$) is the influent mass and the effluent mass from the previous cycle less the mass obtained after the anaerobic phase (3.16).

For the aerated period, the expected values are the values obtained after the anaerobic feeding, because it is the value if there was no removal (3.9 and 3.11). The equations used to calculate the mass variation and the adsorbed mass are similar for the anaerobic feeding phase, but with the appropriate changes (3.13 and 3.15, respectively). The total removed is the difference between the anaerobic feeding mass and the effluent mass (3.17).

Finally, to obtain the percentage removed by adsorption and biodegradation, the mass removed by each process was divided by the total removed (3.18 to 3.21).

3.2.4.3 HPLC analyses

EDCs were analyzed in a chromatograph equipped with a fluorescence detector using a Waters Novapak PAH C18 120 Å column (Waters Corporation). The mobile phase comprised acetonitrile (eluent A) and ultrapure water (eluent B). The run was performed at a constant flow rate of 1 mL min⁻¹. The elution started with a proportion of 40:60 (%) (A:B) for 6 min, followed by 50:50 (A:B) for 3 min, 30:70 (A:B) for 4 min, and 40:60 (A:B) for the last 2 min, totalizing a run time of 15 min. The injection volume of each sample was 20 µL. The retention time for BPA, E2, and EE2 were 6.9, 8.0, and 8.5 min, respectively. For the EDCs liquid phase quantification, a standard curve of each compound in the influent wastewater was prepared. On the other hand, for the quantification of EDCs in sludge mass, a standard curve of each compound in the solid extraction medium (methanol) was prepared, both at concentrations of 5 to 1000 µg L⁻¹, with 7 points of serial dilutions and linearity above 0.99 (R²) for all compounds. The data acquisition and processing of all data was performed in Breeze 2 software (Waters Corporation).

3.2.5 YES assay

The estrogenic activity of the bioreactor samples was performed according to the method of ROUTLEDGE; SUMPTER (1996), with the modifications made by GOMES (2020), using a recombinant receptor gene assay known as the yeast estrogen screen (YES) test. YES consists of an in vitro assay that uses a genetically modified yeast strain of *Saccharomyces cerevisiae*, which synthesizes the enzyme β-galactosidase. The production of this enzyme depends on the amount of active ligands estrogens that bind to yeast cell receptors. The degradation of the chromogenic substrate CPRG (Chlorophenol red-β-D-galactopyranoside), which is added to the assay forms CPR (Chlorophenol red). The quantification of the CPR produced allows, by spectrophotometry, to measure the quantification of β-secreted galactosidase. Thus, the amount of active ligands coupled to the receptors, activating or inhibiting the cellular process, are measured, making it

possible to calculate the estrogenic activity of the analyzed sample (ROUTLEDGE; SUMPTER, 1996).

The YES test was performed along bioreactor operation, using samples collected after anaerobic feeding and at the end of the cycle (effluent). E2 standard curves were prepared in ethanol by a serial dilution of an E2 stock solution ($54.48 \mu\text{g L}^{-1}$) (positive control) to reach concentrations between 2,724 and 1.33 ng L^{-1} . Aliquots of $10 \mu\text{L}$ of each dilution and each sample were transferred to a 96-well optically flat microlitre plate and tested in duplicate. Upon ethanol evaporation, a volume of $200 \mu\text{L}$ of the assay medium (fresh growth medium, recombinant yeast and CPRG) was added to each microlitre plate. The microplates were sealed and shaken vigorously (IKA, model MS-3) for 2 min and then incubated for 72 h at $30 \text{ }^\circ\text{C}$ (Nova Ética 410). Absorbance was measured at 575 nm (for colour) and 620 nm (for turbidity) in a Versa Maax spectrometer microplate reader (Molecular Devices).

The estrogenic activity present in the samples was expressed as estradiol equivalent (EQ-E2), calculated by interpolating the curve data from the E2 standard curve. The dose-response curves were fitted to a symmetric logistic function using the Origin 6.0 software package (Microsoft, USA).

3.2.6 Analytical methods

COD, phosphate, ammonium, nitrate, and nitrite concentrations were determined by the colorimetric method (APHA, 2005) after sample filtration through nitrate cellulose (0.45 mm pore size). The absorbance was measured in a Hach DR3900 spectrometer (Hach Instrument). Total suspended solids (TSS) and volatile suspended solids (VSS) within the reactor were quantified according to the methodology described by Pronk *et al.* (2014). Extracellular polymeric substances (EPS) were extracted from the granular biomass by heating method (BASSIN *et al.*, 2012) and quantified as protein (PN) and polysaccharides (PS) using the methods proposed by BRADFORD (1976) and DUBOIS *et al.* (1956), respectively. Besides evaluating the reactor performance based on influent and effluent concentrations of different compounds, their concentration profiles over the SBR cycle were also assessed by collecting samples at pre-defined time intervals. These experiments were referred to as cycle tests.

3.2.7 DNA extraction, 16S rRNA gene sequencing and bioinformatic analysis

DNA was extracted from approximately 20 mg of wet granular sludge samples collected from the reactor at different experimental phases using the PureLink Microbiome DNA Purification kit (Thermo Fisher Scientific, USA), with two runs in a FAST PREP-24 5G (MP Biomedicals, USA) at 6.0 m s^{-1} for 40 s for cell lysis. DNA quality was assessed through 0.8% agarose electrophoresis, and DNA concentration was estimated with a Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Extracted DNA was subjected to sequencing of the V4 region of the 16S rRNA gene by the Novogene company (USA). Sequencing was done in an Illumina NovaSeq 6000 (Illumina, USA) with 100K paired-end reads, using the primers 515F and 806R (GTGCCAGCMGCCGCGGTAA; GGACTACHVGGGTWTCTAAT) (CAPORASO *et al.*, 2011). Fastq files from the sequencing run had their primers and barcodes removed by the company, and they were further analyzed with Mothur v.1.46.1 software (SCHLOSS *et al.*, 2009). Forward and reverse paired sequences were joined into contigs, and sequences containing ambiguities (N-base), more than 8-mer homopolymers, and unexpected sizes were removed. Identical sequences were grouped and aligned with the SILVA 138 SSU database (QUAST *et al.*, 2012), after a virtual PCR in the database with 515F-806R primers. Then, non-overlapping sequences were removed by defining the boundaries of the alignment. A pre-cluster was performed to merge rare sequences into abundant ones, with a threshold of 2 bp. Chimeric sequences were removed through chimera.vsearch (ROGNES *et al.*, 2016). Sequences were then classified using the RDP 18 database (COLE *et al.*, 2009) also after a virtual PCR, with an 80% of cutoff. Reads classified as mitochondria, chloroplasts, Archaea, Eukarya, or unknown were removed. The sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold, and singletons were removed. Samples were subsampled to the lowest common number of sequences amongst them (133,164). Alfa diversity indexes, taxonomical composition, and the OTU distribution were exported.

A non Metric Multidimensional Scaling (nMDS) and a neighbor-joining tree using Bray Curtis dissimilarity index were conducted in Past4.08 software (HAMMER *et al.*, 2001). Raw data were deposited in NCBI Sequence Read Archive (SRA) under Bioproject PRJNA887127 and Biosamples SAMN31155807 to SAMN31155812.

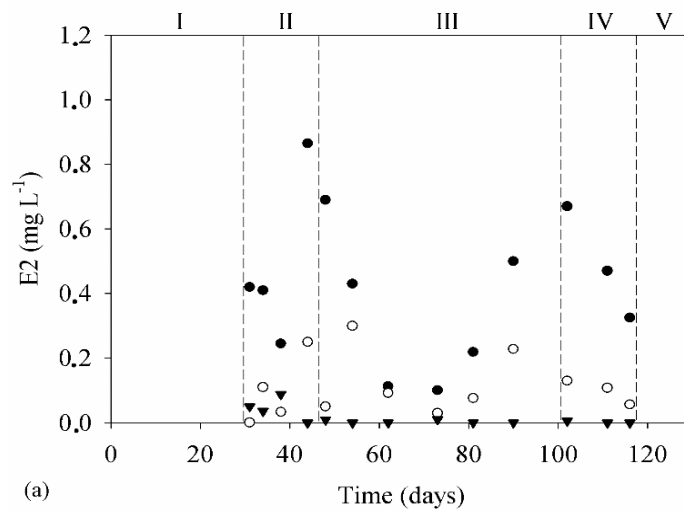
3.3 Results and discussion

3.3.1 EDCs removal

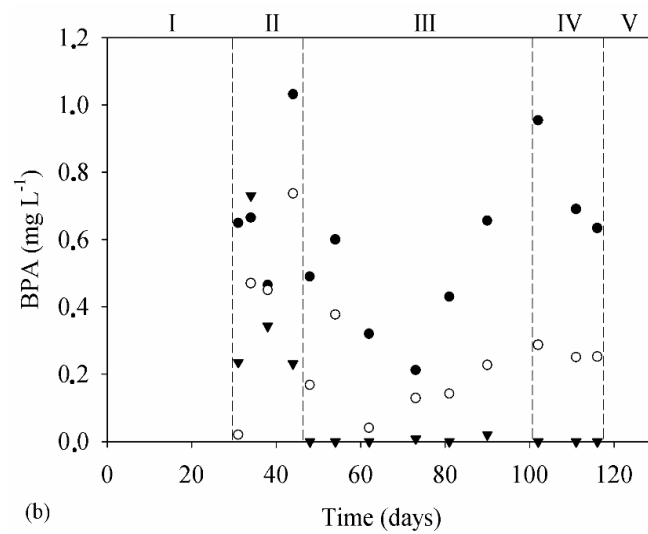
3.3.1.1 Compounds quantification in the bulk liquid

The mixture of E2 and BPA was fed to the reactor starting from day 31 to day 44 (phase II). Before that, the reactor was fed with synthetic wastewater without EDCs for 30 days. On the 45th day of reactor operation, EE2 was also added to the influent (phase III), and it was removed from the system on day 102 (phase IV). The concentration of each compound during a cycle throughout the operating days is shown in Figure 3.1.

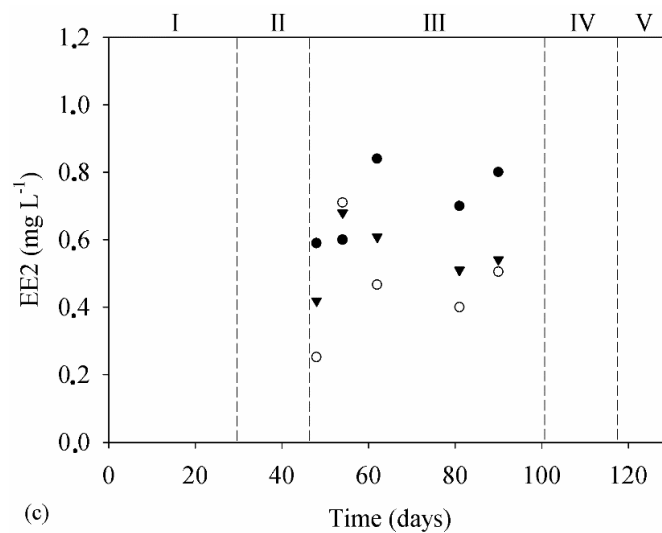
The influent concentration of E2 varied between 0.1 and 0.9 mg L⁻¹, with an average value of 0.45 mg L⁻¹ over the entire operation time. During phase II, the average E2 concentration in the effluent was 0.044 mg L⁻¹. However, from phase III onwards, E2 concentration was below the detection limit in the outlet stream (Figure 3.1a). Through the quantification of the compounds during a complete SBR cycle (Figure 3.2), a considerable amount of E2 was found to be consumed in the anaerobic feeding period, with the remaining being consumed within 40 min of the aerated stage. Figure 3.3a shows that on the first day, practically all E2 was already consumed in the anaerobic phase. However, considering all SBR operating phases, an average removal of 55% was attained anaerobically, while the removal during aeration was 42%.



(a)



(b)



(c)

Figure 3.1 - E2 (a), BPA (b), and EE2 (c) concentration profiles throughout the operating days. Concentrations in influent (●), after anaerobic feeding (○), and effluent (▼) are shown over time.

The influent concentration of BPA ranged between 0.21 and 1.0 mg L⁻¹, with an average value of 0.6 mg L⁻¹ (Figure 3.1b). During phase II, higher BPA concentrations were found in the effluent, sometimes even higher than those in the influent, due to the remaining of the concentration in the effluent from the previous cycle. Besides that, BPA was not completely consumed aerobically, and 55% of the initial concentration remained in the effluent. However, in phases III and IV, BPA concentration significantly decreased during the anaerobic period, and it was completely removed after 40 min within the aerated period (Fig. 3.3). As observed for E2, 95% of BPA was consumed anaerobically on the first day. However, throughout phase II, this removal dropped to 31.7% and subsequently to 0%, while the BPA removal percentage under aeration increased from 0 to 80% (Figure 3.3b). From phase III onwards, BPA removal increased in both stages of the SBR cycle, with the aeration phase contributing the most. Overall removal reached 98.4% (phase III) and 100% (phase IV). From the results obtained, it is possible to infer that BPA is not readily metabolized by microorganisms, and an adaptation time to this compound is needed prior to biodegradation, as reported in previous studies (CYDZIK-KWIATKOWSKA *et al.*, 2017). ELY *et al.* (2022) observed that BPA removal was low in an AGS-SBR system, but improved after bioaugmentation with an EDCs-degrading bacteria.

The EE2 was added to the influent only during phase III, and the concentration profiles over the SBR operating days are shown in Figure 3.1c. The influent EE2 concentration varied between 0.6 and 0.85 mg L⁻¹, with an average value of 0.71 mg L⁻¹. EE2 was detected in all effluent samples, and its concentrations were many times higher than those after the anaerobic feeding. Moreover, its mass fluctuated over the aerated period (Figure 3.2). The main EE2 removal was observed in the anaerobic feeding, except on day 54. (Figure 3.3c). This increase implies that EE2 was released from the granular biomass to the bulk liquid, probably due to desorption. Similar results were also observed in a previous study (ELY *et al.*, 2022), which suggested that EE2 was sorbed onto the granular biomass anaerobically, but desorbed in subsequent SBR cycles, even when the compound was not present in the reactor feeding. Evaluating an upflow anaerobic packed bed biofilm reactor (UAnPBBR) and an aerobic trickling filter biofilm reactor (TF), LIN *et al.* (2020) suggested that the initial removal of EE2 (500 µg L⁻¹) from the liquid phase was likely due to sorption processes that eventually saturated the adsorption sites. EE2 concentration was only reduced by 1.62% in the UAnPBBR, while the TF reached 20.36% removal of this compound. Moreover, EE2 was not readily biodegraded by either

anaerobic or aerobic biofilms at the HRT employed in their study (1.21 days). In a biological activated carbon (BAC) system comprising heterogeneous microbial communities, EE2 was removed mainly by adsorption, with biodegradation slightly contributing to the overall performance (TRAN *et al.*, 2020). These results emphasize that EE2 is less susceptible to microbial degradation than E1 and E2.

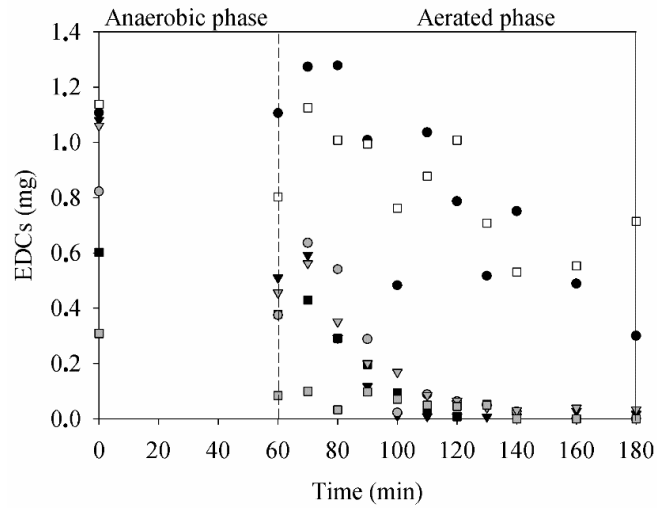
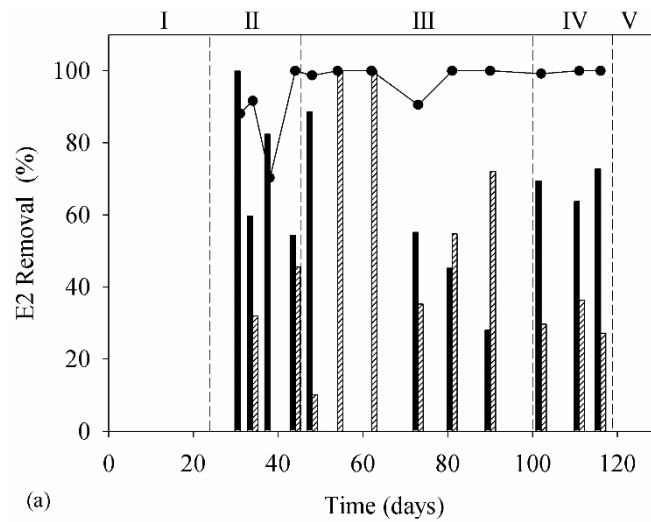
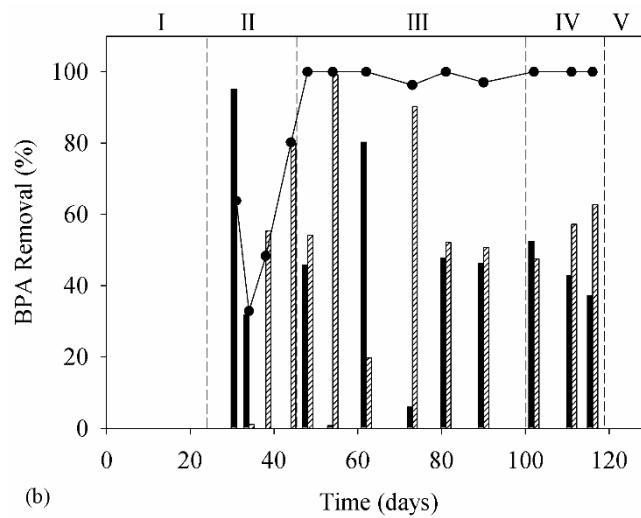


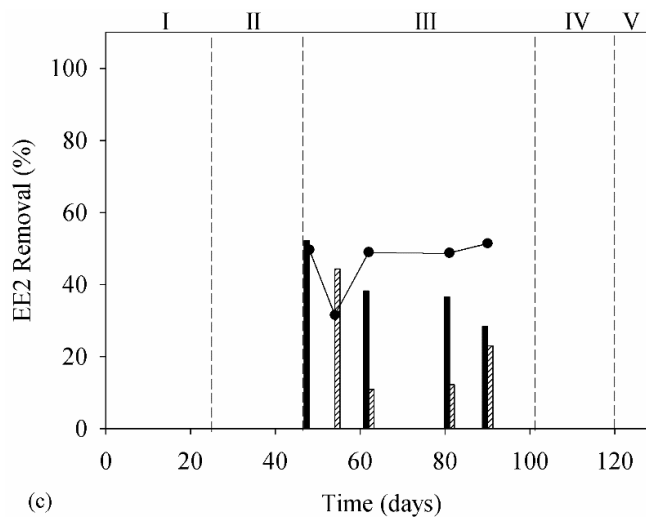
Figure 3.2 - EDCs mass profiles during the SBR cycle: E2 (grey), BPA (black), and EE2 (white). Symbols: 34th day (phase II) (●), 100th day (phase III) (▼), and 116th day (phase IV) (■). The concentration of each compound at time 0 was estimated taking into account the concentration of the compound in the liquid remaining from the previous cycle and the influent stream.



(a)



(b)



(c)

Figure 3.3 - E2 (a), BPA (b), and EE2 (c) removal efficiency in each stage of the SBR cycle (anaerobic feeding and aeration): anaerobic removal (black bar), aerobic removal (hatched bar), and overall removal efficiency (●).

3.3.1.2 Compounds quantification in the granular biomass

To better understand the mechanisms underlying EDCs removal, the target compounds were extracted from the granular biomass at the end of the anaerobic feeding and the end of the sedimentation time. The contribution of adsorption and biodegradation to EDCs removal in both anaerobic and aerated SBR cycle phases was then estimated, taking into account the expected amount of each compound that would be detected if there was no removal and the measured content (Figure 3.4).

To assess the mass of each compound adsorbed on the granular biomass during the anaerobic feeding of a certain cycle, it is necessary to consider the mass adsorbed in the immediately preceding cycle. This is because the sludge at the end of the cycle is the same as that at the beginning of the next cycle (the amount of sludge wasted by effluent withdrawal and manual discharge were considered negligible and therefore not accounted for).

In the first cycle after the addition of EDCs, 99.9% of E2 was consumed in the anaerobic feeding, 50.2% being adsorbed and the remaining biodegraded. During the entire experimental period, removal of E2 was observed within the anaerobic feeding period, part of it being adsorbed and the other biodegraded. In phase IV, the biodegradation was more pronounced (80.9%) than the adsorption (19%) (Figure 3.4a). Over the aerated period, the amount of E2 in the granular biomass decreased, which means that part of this compound that was adsorbed during anaerobic feeding was released to the bulk liquid. This could be sorbed again and then metabolized by the microorganisms. However, only from day 44 onwards, reduction of E2 content began to be observed within the aerated period, with biodegradation being the only mechanism for its consumption.

Like E2, BPA was totally consumed in the anaerobic feeding within the first cycle after EDCs supplementation, being 64.8% sorbed on the granular biomass and 35.2% biodegraded. This is expected since the active sites were all free. However, this was not observed in the following two analyses on days 34 and 38. From day 44 to day 73, almost 100% of BPA was adsorbed into the granular biomass during the anaerobic feeding. Nevertheless, from day 81 onwards, the role of adsorption became marginal, and the removal of BPA attributed to this process decreased from 84.6% (day 81) to 1.1% (day

116). On the other hand, the removal via biodegradation increased from 15.4% (day 81) to 98.9% (day 116). During the aerated period, adsorption was noticed only in the second and third analyses carried out on days 34 and 38, respectively. From the 44th day onwards, BPA was completely biodegraded. In fact, part of the BPA content that was sorbed during the anaerobic period was released into the bulk liquid in the aerated period, being then biodegraded (Figure 3.4b).

Regarding EE2, as discussed previously, this compound was hardly removed. During the anaerobic period, a portion of EE2 (5.1 to 22%) was sorbed onto granular biomass, while the remaining amount that was removed was attributed to the metabolization of microorganisms. In the subsequent aerated period, part of EE2 sorbed onto granular biomass anaerobically returned to the bulk liquid. The bacterial metabolization was the main mechanism responsible for reducing EE2 concentrations in the liquid, although only a small fraction of EE2 was biodegraded.

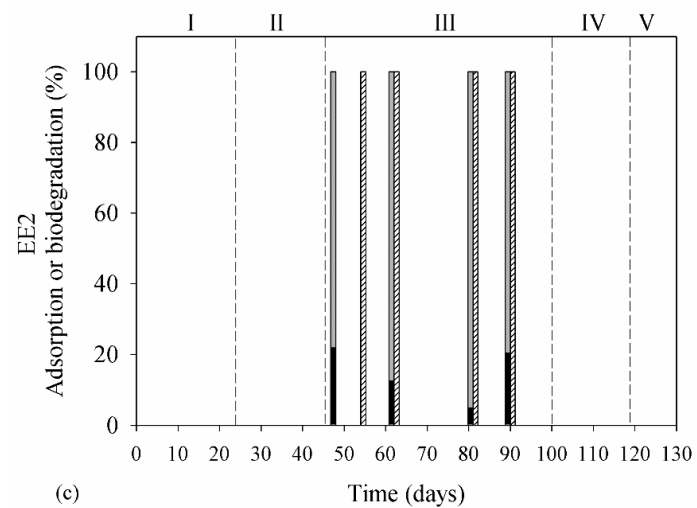
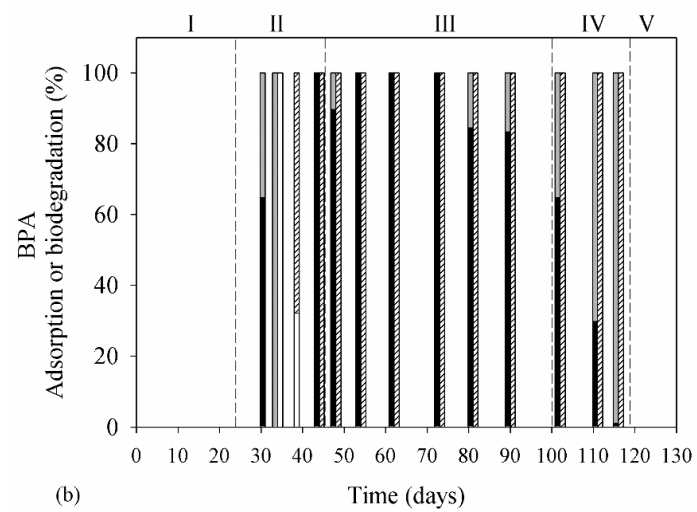
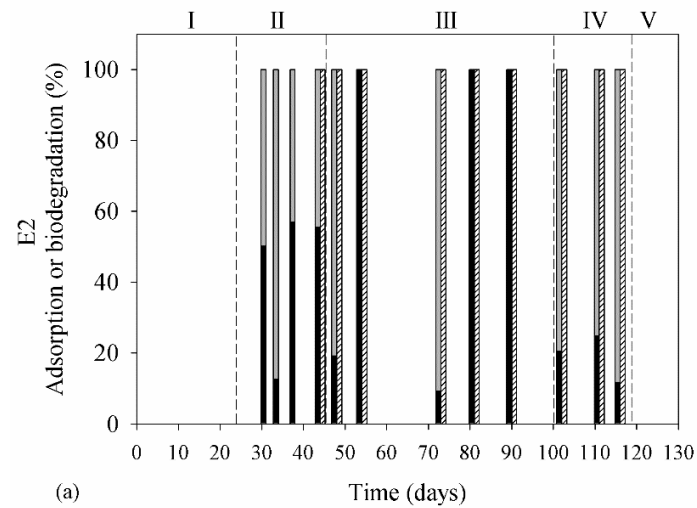


Figure 3.4 - Percentage contribution of adsorption and biodegradation for the overall removal of E2 (a), BPA (b), and EE2 (c) achieved in each period of the SBR cycle. Removal by adsorption (black bar) and biodegradation (grey bar) during anaerobic feeding and removal by adsorption (white bar) and biodegradation (hatched bar) during

the aerated phase. Operating days with only one bar means that there was no removal at some phase of the SBR cycle (either anaerobic or aerated).

3.3.1.3 Estrogenic activity

Estrogenic activity was assessed by YES assay from samples regularly collected after anaerobic feeding and at the end of the cycle (effluent). It was expressed as equivalents of E2 (E2-EQ). E2-EQ compares the estrogenic potential of the analyzed samples with the estrogenic potential of E2. The toxic effects of some samples might inhibit the full growth of yeast cells and, consequently, the estrogenic activity can be masked, decreasing the response observed in the assay. For samples that show cytotoxicity, the interference could be overcome by series dilution (GOMES, 2020). Nevertheless, even after the series dilution, 8.3% and 12.5% of the samples from phases II and III, respectively, presented cytotoxicity and, therefore, were not used for calculating the average of E2-equivalents, as shown in Figure 3.5.

Given that in this study the influent concentrations of target compounds (EDCs) were in the order of mg L^{-1} , estrogenic activity was expected to be present in the samples. Most previous research on biological treatment of EDCs-containing wastewaters addressed the YES assay only in effluent samples. However, part of the estrogens can be removed under anaerobic conditions, as previously reported. Therefore, the estrogenic activity after the anaerobic feeding phase of the SBR was also evaluated. The results showed a significant reduction in estrogenic activity within the first 60 min of feeding, with a removal percentage of 64.2% and 57.9% for phases II and IV, respectively. For the samples collected at the end of the cycle, the reduction in estrogenic activity was even higher, reaching 81.8% (phase II) and 96.5% (phase IV). Furthermore, it is worth mentioning that the decrease in estrogen activity after the aeration period was more evident (10 times higher) at the end of the reactor operation time (phase IV) than at the beginning (phase II). This result indicates that the long-term operation enhances granular sludge capability in estrogenicity reduction.

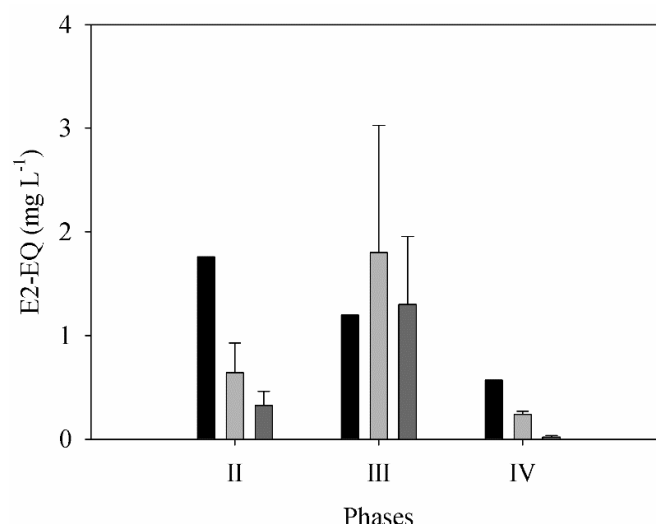


Figure 3.5 - Estrogenic activity given as E2-equivalents (E2-EQ) for the following samples: influent (black bar), after the anaerobic feeding (light grey bar), and effluent (medium grey bar). For the influent, only one analysis per phase was performed because the wastewater composition remained constant in a certain phase.

In contrast, during phase III, when EE2 was also supplemented to the influent, the average estrogenic activity of the samples collected after the anaerobic feeding and at the end of the cycle (effluent) was higher than that obtained for the influent. This behavior may result from the release of this compound sorbed in the biomass during previous cycles, as explained before. EE2 has been considered the most biologically active estrogen, exhibiting a 1.25-fold higher estrogenicity than E2 (BEEK *et al.*, 2006). In contrast, even detecting EE2 in the effluent of an AGS-SBR fed with 20 $\mu\text{g L}^{-1}$ of this compound, CASTELLANOS *et al.* (2021a) found an average residual effluent estrogenic activity of only 0.09 $\mu\text{g L}^{-1}$ EQ-E2.

3.3.2 COD, P and N removal

Before EDCs addition, the AGS-SBR was only fed with synthetic domestic wastewater without EDCs for 30 days, enough time for the reactor to reach steady-state conditions, with C, N and P conversions stable over time.

During the entire experiment, the AGS-SBR exhibited a stable COD removal efficiency, reaching effluent COD always lower than 100 mg L^{-1} , mostly varying from 35 to 60 mg L^{-1} (Figure 3.6a). Only at the beginning of phase II (E2 and BPA added to the

influent stream) and phase III (addition of EE2) there was a slight decrease in the COD removal performance. The majority of the influent organic matter was consumed in the anaerobic feeding, when no external electron acceptors were provided. This conversion can be performed by polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs), whose metabolism does not depend on an external electron acceptor to convert the organics to intracellular polymers (namely polyhydroxyalkanoates – PHA) (BASSIN, 2018). The latter are then used as a carbon source in the subsequent aerated phase of the SBR for both phosphate uptake by PAOs and glycogen replenishment by GAOs (De KREUK *et al.*, 2004). They also serve as an electron donor for denitrification by denitrifying PAOs (DPAOs) and GAOs (DGAOs) within the anoxic zone of the granules (NANCHARAI AH; REDDY, 2018).

However, during phases II and III, there was a greater availability of organic matter in the aerobic period (COD of 160 mg L⁻¹ for both phases) compared to phase I (COD of 91 mg L⁻¹). From phase IV onwards, when EE2 was removed from the influent, almost full organic matter consumption was observed in the anaerobic period, and only 10.7% (phase IV) and 5.2% (phase V) of incoming COD were removed within the aerated period. The remaining COD in the effluent, about 38 mg L⁻¹, was due to the presence of non-biodegradable organic matter, partially present in the influent and partially formed from the by-products resulting from microbial degradation. This fraction would not be consumed even by prolonging the duration of the SBR cycle.

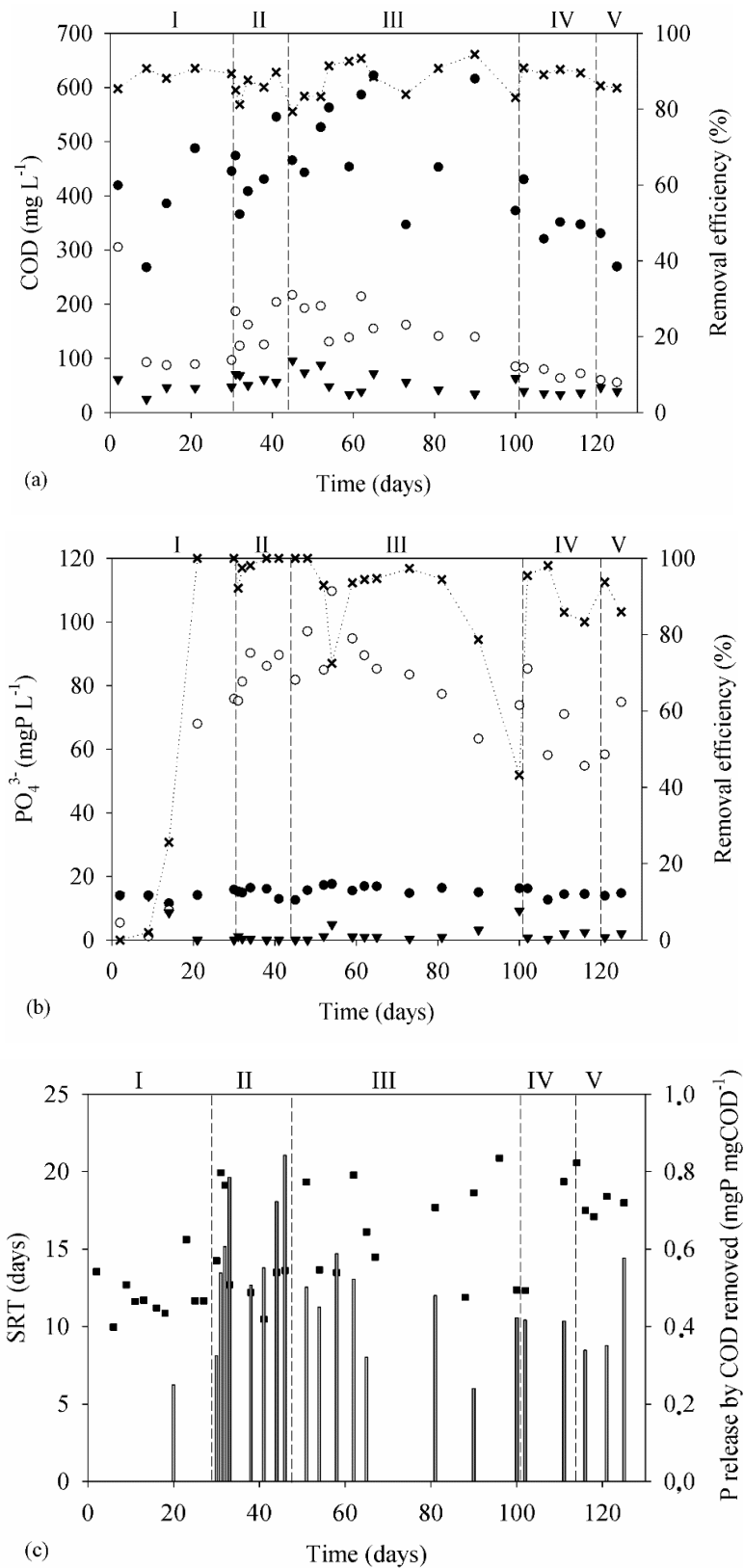


Figure 3.6 - COD (a), PO₄³⁻-P (b) concentrations and P released by COD removed with SRT (c) over the AGS-SBR operation time. Concentrations were measured in the influent (●), after the anaerobic feeding (○), and effluent (▼). The removal efficiency (×) was

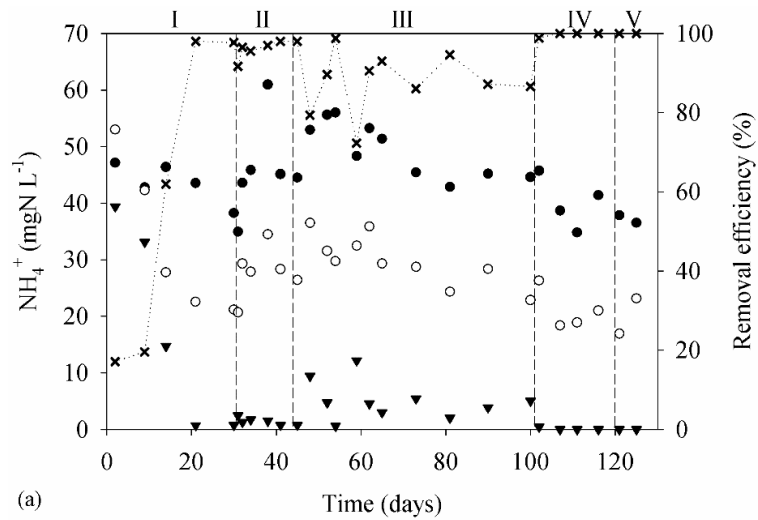
calculated from the concentration in influent and effluent samples. SRT (■) and P released by COD removed (gray bar) are also shown on the left and right y-axis, respectively.

The phosphate concentration profiles throughout the reactor operation time are shown in Figure 3.6b. Initially, during reactor start-up, the phosphate concentration in the influent and effluent was the same. This is because the activity of PAOs takes some time to be restored. From 21 days onwards, after the anaerobic feeding, phosphate levels were consistently higher than in the influent. This result is associated with the release of PO_4^{3-} -P by PAOs under anaerobiosis (De KREUK; Van LOOSDRECHT, 2004). However, the amount of PO_4^{3-} -P released by COD removed anaerobically gradually decreased during phase III, and consequently, the PO_4^{3-} -P removal during aeration also dropped (Fig. 3.6.c). On day 100, phosphate concentration in the effluent reached the highest value (9.2 mgP L^{-1}), with the removal efficiency decreasing to 43.2%. A reduction in anaerobic P-release in the presence of emerging contaminants was also observed by AMORIM *et al.* (2016) and KENT; TAY (2019). However, as reported by these authors, the phosphate concentration in the effluent was not affected. On the other hand, some studies reported adverse effects on phosphate removal by aerobic granules in the presence of estrogens (CASTELLANOS *et al.*, 2021; ELY *et al.*, 2022). After removing EE2 from the influent (phase IV), phosphate removal was recovered, reaching up to 98%, despite the presence of E2 and BPA in the influent stream. This result suggests that EE2 adversely affected PAOs activity, given that the operating conditions of the SBR (including the sludge age, controlled between 10 and 20 days) were similar in all phases.

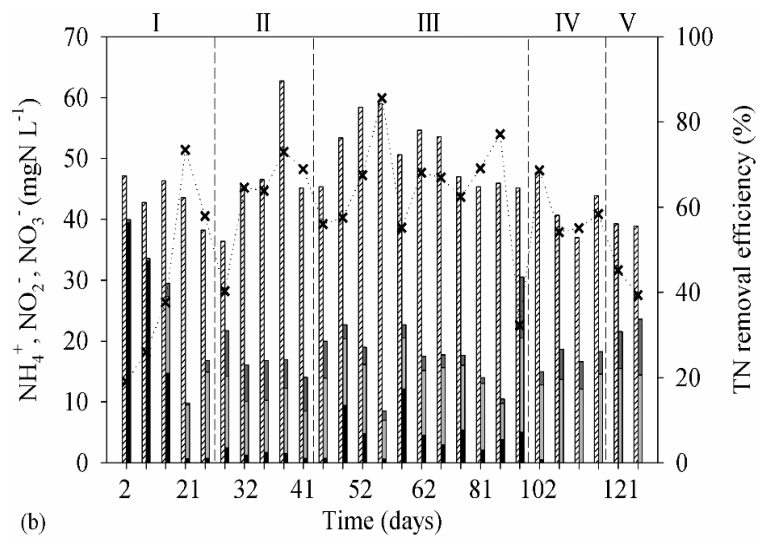
Figure 3.7a illustrates the ammonium concentration profiles over the reactor operation time. During the first days of SBR operation, after inoculation, nitrification levels gradually increased until day 21 (phase I). After that, the ammonium concentration in the effluent reached below 1 mgN L^{-1} on day 30. In phase II, upon the addition of E2 and BPA, the average effluent ammonium increased to 1.5 mgN L^{-1} . This indicates a slight adverse effect of these compounds on nitrification, but long-term acclimation allowed to improve ammonium removal. When EE2 was added to the influent stream along with the other estrogens (phase III), there was a significant increase in effluent ammonium concentrations, reaching up to 12 mgN L^{-1} . Partial inhibition of the nitrification process in AGS was reported by CASTELLANOS *et al.* (2021) at $20 \text{ }\mu\text{g L}^{-1}$ of EE2 and E2. In phase IV, after removing EE2 from the influent, ammonium removal

efficiency quickly attained 100%, implying a fast recovery of the ammonium-oxidizing bacteria (AOB) activity. Complete ammonium removal remained so until the end of the experiment. As observed in the PAOs activity, EE2 adversely affected AOB activity.

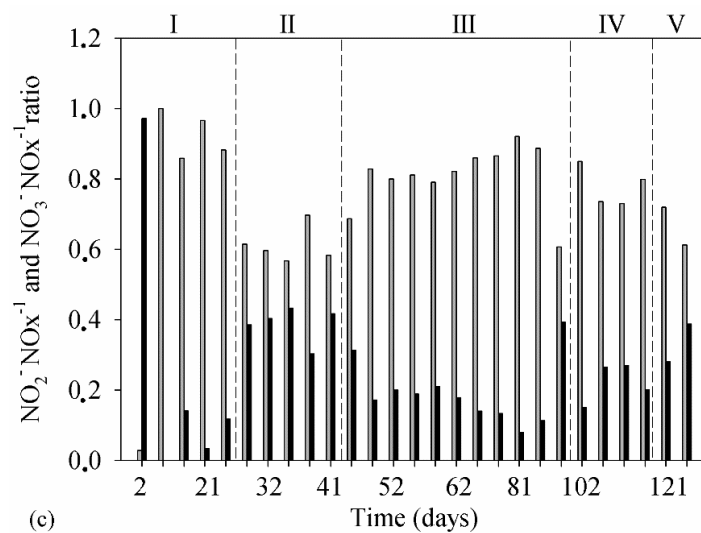
To assess the overall nitrogen balance, the main products of nitrification, nitrite and nitrate, were also analyzed over the operation time. Each oxidized nitrogen compound is displayed in Figure 3.7b, along with the total nitrogen (TN) removal. Nitrite was the main nitrification product in all phases, except in phase II, as shown in Figure 3.7c. It was found in relatively high concentrations in the effluent, between 7.0 to 15.0 mgN L⁻¹. NOB activity impairment and consequent nitrite accumulation has been shown to be associated with the presence of EE2. However, the structure of nitrifying granular sludge could reduce the negative effects of EE2 on AOB, which might degrade EE2 via cometabolism (WANG *et al.*, 2021). Regarding nitrate, during phase I, the average nitrate concentration in the effluent was 0.96 mgN L⁻¹. After that, there was a decrease in its average effluent concentration from 6.0 mgN L⁻¹ (phase II) to 1.83 mgN L⁻¹ (phase III), returning to 7.5 mgN L⁻¹ in phase V (Figure 3.7b). Total nitrogen removal dropped from 65.7% (phase I, after the nitrification process was restored) to 42.3% (phase V) mainly due to the increment in the concentration of nitrification products.



(a)



(b)



(c)

Figure 3.7 - $\text{NH}_4^+\text{-N}$ concentration profiles (influent (\bullet), after the anaerobic feeding (\circ), effluent (\blacktriangledown)) (a); all inorganic nitrogen species ($\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$) (influent – NH_4^+ (hatched bar), effluent - NH_4^+ (black bar), effluent – NO_2^- (light grey bar), effluent

– NO_3^- (dark grey bar) and TN removal efficiency (\times) (b); and $\text{NO}_2^-/\text{NO}_x^-$ (grey bar) and $\text{NO}_3^-/\text{NO}_x^-$ (black bar) ratios (c) over the AGS-SBR operating time.

Cycle tests were carried out during the last days of each phase (except phase V) to assess the dynamics of several compounds over the operating cycle (Figure 3.8). Around 70% (phase I), 66% (phase II), 77% (phase III) and 79% (phase IV) of the incoming COD was removed in the anaerobic feeding period, remaining almost constant until the end of the cycle, except for phase II. Ammonium was not fully oxidized in phases II and III, but was completely removed within 160 min of the SBR cycle in phase IV. Nitrite levels increased throughout the cycle, showing effluent concentrations of 7.85 (phase I), 15.09 (phase II), 15.48 (phase III), and 14.59 mgN L^{-1} (phase IV). The same pattern of increasing throughout the cycle was observed for nitrate; however, in phases I and IV, the effluent NO_3^- levels were lower (4.37 and 3.68 mgN L^{-1}) compared to phases II and III (9.99 and 10.04 mgN L^{-1}). Regarding the EBPR process, it was observed that phosphate release was similar under anaerobic conditions for all phases. However, P removal was complete only in phase I, and P removal efficiency decreased during the following experimental phases. The specific ammonium, phosphate and NO_x^- (denitrification) removal rates are shown in Table 3.3. During phases II and III, denitrification rates were lower than the phases I and IV, indicating that this process was impaired by the presence of EDCs. Besides that, the specific phosphate uptake rate decreased throughout the phases.

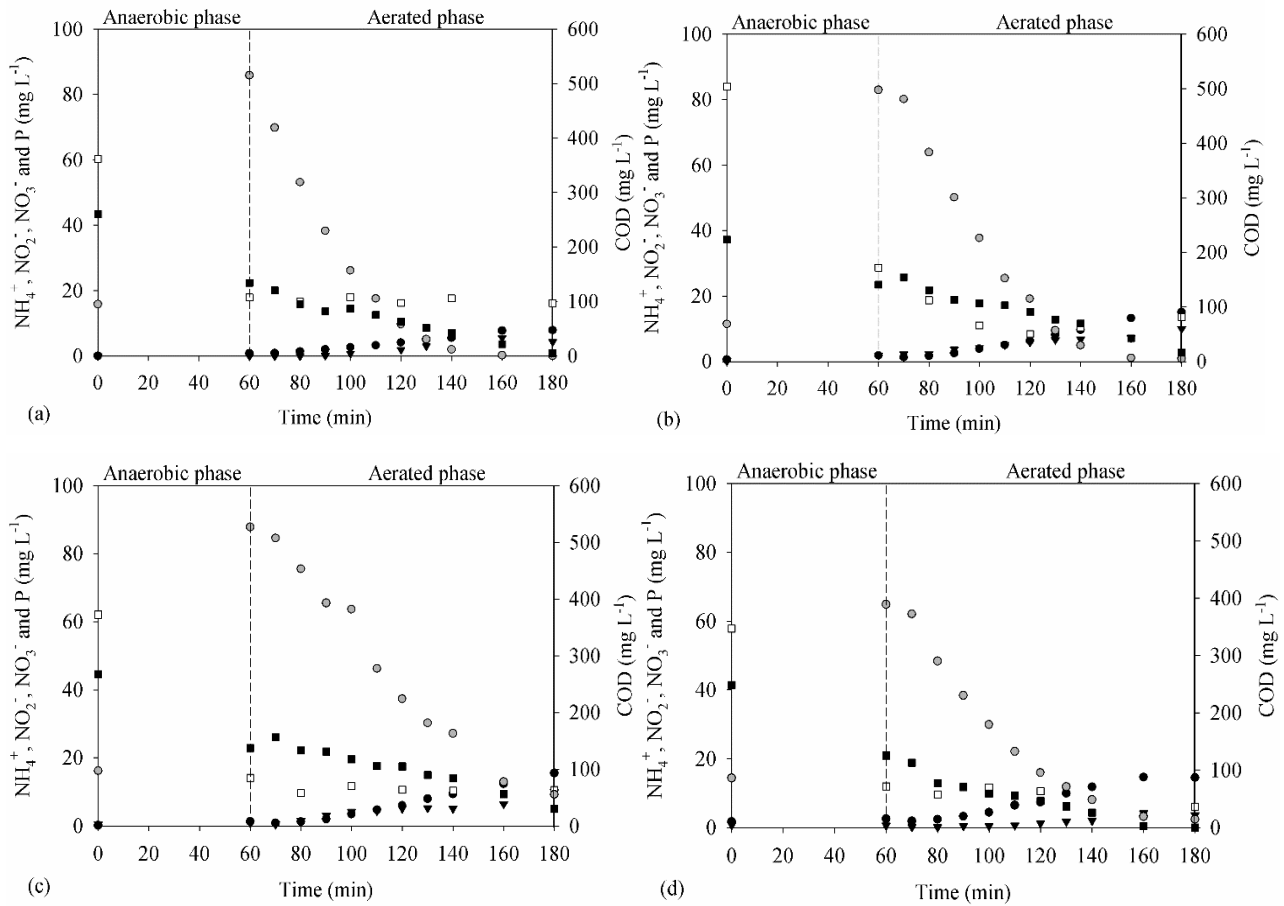


Figure 3.8 – COD and nutrients profiles over the SBR cycle: (a) phase I, (b) phase II, (c) phase III and (d) phase IV. Symbols: COD (\square), NH_4^+ (\blacksquare), NO_2^- (\bullet), NO_3^- (\blacktriangledown), P (\circ).

Table 3.3 – Specific NH_4^+ , NO_x^- and PO_4^{3-} removal rates obtained in the cycle test carried out under stable SBR operating conditions.

Rates	Phase I	Phase II	Phase III	Phase IV
NH_4^+ (mgN (gSSV.h)^{-1})	1.32	0.90	0.83	0.90
Denitrification (mgN (gSSV.h)^{-1})	0.38	0.16	0.22	0.49
PO_4^{3-} (mgP (gSSV.h)^{-1})	6.82	4.58	3.43	2.51

3.3.3 Physical characteristics of granular biomass

The granular biomass exhibited a compact structure throughout the experiment, with light yellow. The granules size distribution and the mean diameter in different experimental phases are shown in Figure 3.9. Particles with size lower than 0.2 mm were

marginal and could not be sustained in the reactor during the short settling time before the withdrawal phase. Diameters between 0.2 and 2.0 mm increased throughout the phases, which resulted in a decrease in the mean diameter from 2.6 mm to 2.0 mm, indicating that EE2 supplementation also affected the granules size distribution.

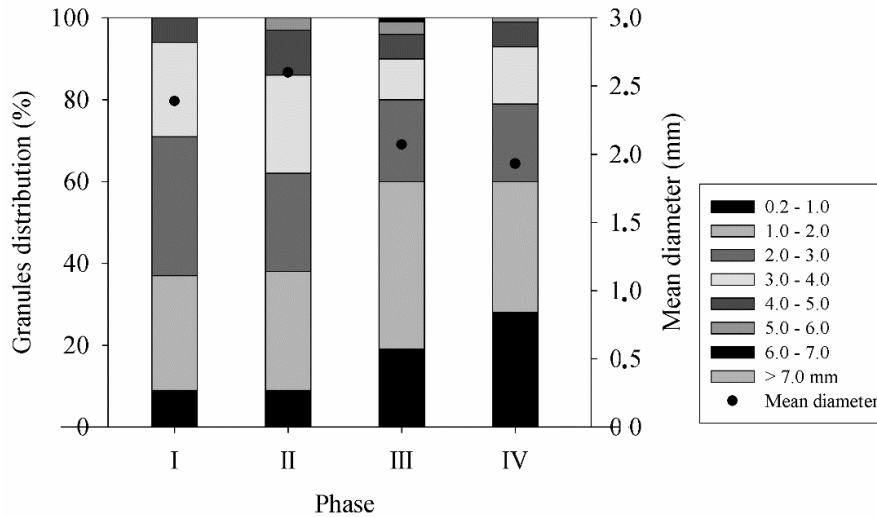


Figure 3.9 - Granules size distribution and mean diameter (mm) for phases I, II, III and IV.

The solids content inside the reactor changed little after phase I (Figure 3.10a). During phase I, both TSS and VSS concentrations gradually increased from 1.13 to 10.94 gTSS L⁻¹ and 0.94 to 7.8 gVSS L⁻¹, respectively. However, after EDCs addition (phase II), TSS and VSS concentrations decreased progressively until day 38 and increased again on day 41 to values close to those observed on day 30 (11.4 g TSS L⁻¹ and 8.10 g VSS L⁻¹). During this phase, the VSS TSS⁻¹ ratio slightly decreased from 0.71 to 0.65. In phases IV and V, VSS TSS⁻¹ ratio remained virtually unchanged. It is important to mention that the SRT was maintained between 15 to 20 days (Fig. 3.6c) by manual biomass discharge and natural suspended solids washout. The latter was accounted for as solids in the effluent (Figure 3.10b). The decrease in the solids inside the reactor during phase II was reflected in the increase in the TSS and VSS in the effluent, indicating that small particles (size lower than 0.2 mm) were possibly washed out during effluent withdrawal. A significant increase in the effluent solids content was again observed only at the beginning of phase III, but it dropped again in the subsequent phases.

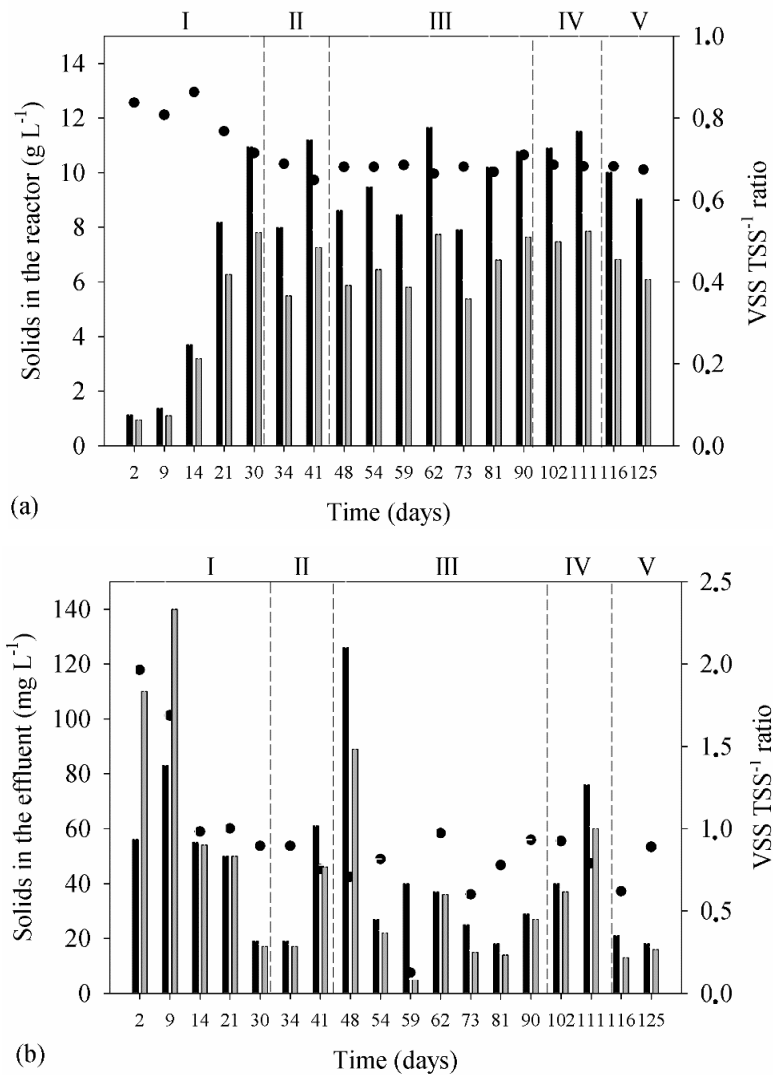


Figure 3.10 - Solids content inside the reactor, with the respective VSS TSS⁻¹ ratio (a) and the solids in the effluent (b). Legend: TSS (black bar), VSS (grey bar), VSS TSS⁻¹ ratio (●).

The EPS content of the granular biomass was characterized in terms of proteins (PN) and polysaccharides (PS) (Figure 3.11). In general, the EPS content increased from the beginning to the end of the AGS-SBR operation time, with average PN and PS concentrations of 240 to 533 mgPN gVSS⁻¹ and 54 to 104 mgPS gVSS⁻¹, respectively. Production of EPS has been reported as a protection mechanism by microorganisms against contact with toxic substances. CASTELLANOS *et al.* (2021) reported an increment in PN and PS contents upon estrogens (E2 and EE2) to an AGS system and a subsequent decrease when the system achieved stability. In this study, however, even after EDCs removal from the feeding (phase V), the EPS content continued to increase.

It was noticed that the PN PS^{-1} ratio increased considerably in phases II (6.96) and III (7.00) in the presence of EDCs, but returned to values close to that observed in phase I (i.e., 4.41) after EE2 (5.30 – phase IV) and E2 and BPA (5.11 – phase V) had been removed from the incoming stream. EPS production has been reported to be a strategy of microorganisms to protect themselves against external stress, and some hydrophobic regions of the exopolymers increase the adsorption capability of several organic pollutants (SHENG *et al.*, 2010).

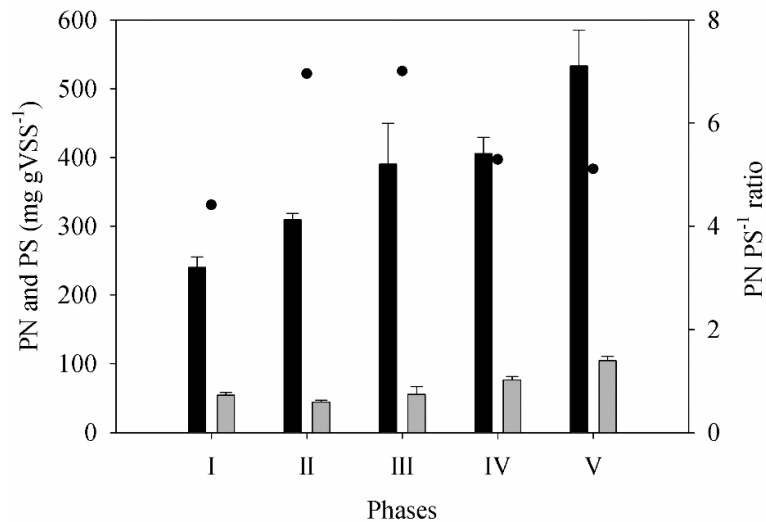


Figure 3.11 - Protein (black bar) and polysaccharides (grey bar) content in the granular biomass, and PN PS^{-1} ratio (●).

3.3.4 Microbial community of the aerobic granules

The microbial community in all samples collected during AGS reactor operation was mainly affiliated to 5 phyla (abundance >1%), being one of them assigned to unclassified sequences. Proteobacteria (77.5% of all sequences), followed by Bacteroidetes (11.2% of all sequences) were the most dominant phyla, consistent with previous studies addressing AGS systems (WANG *et al.*, 2021; XIA *et al.*, 2018; ZHAO *et al.*, 2015).

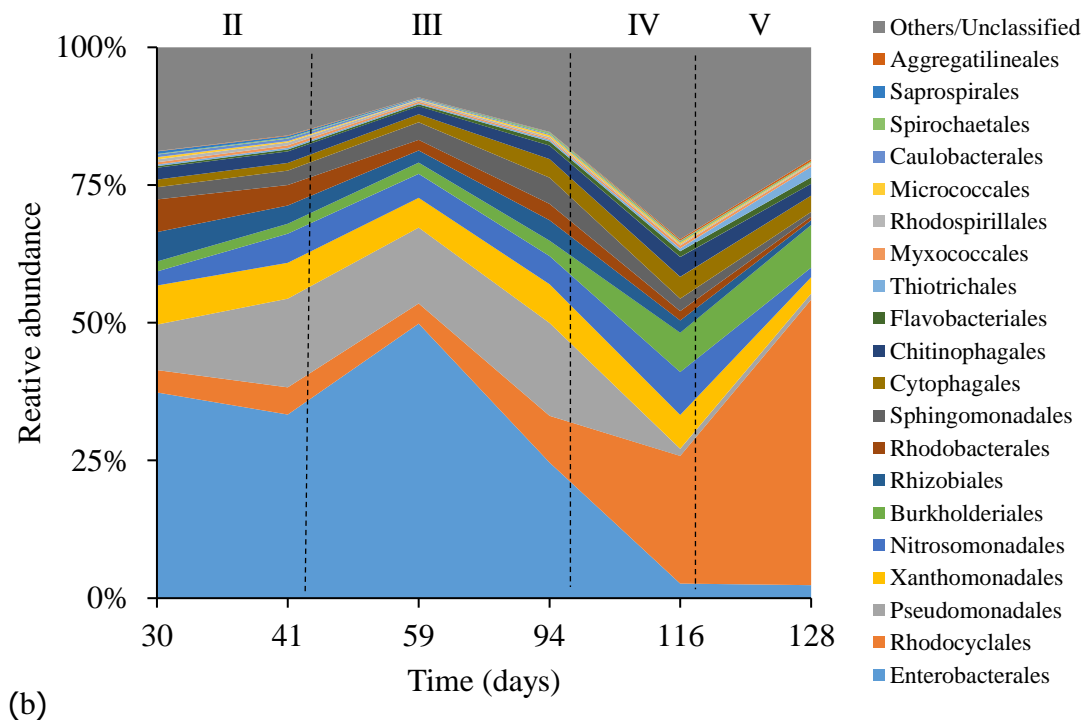
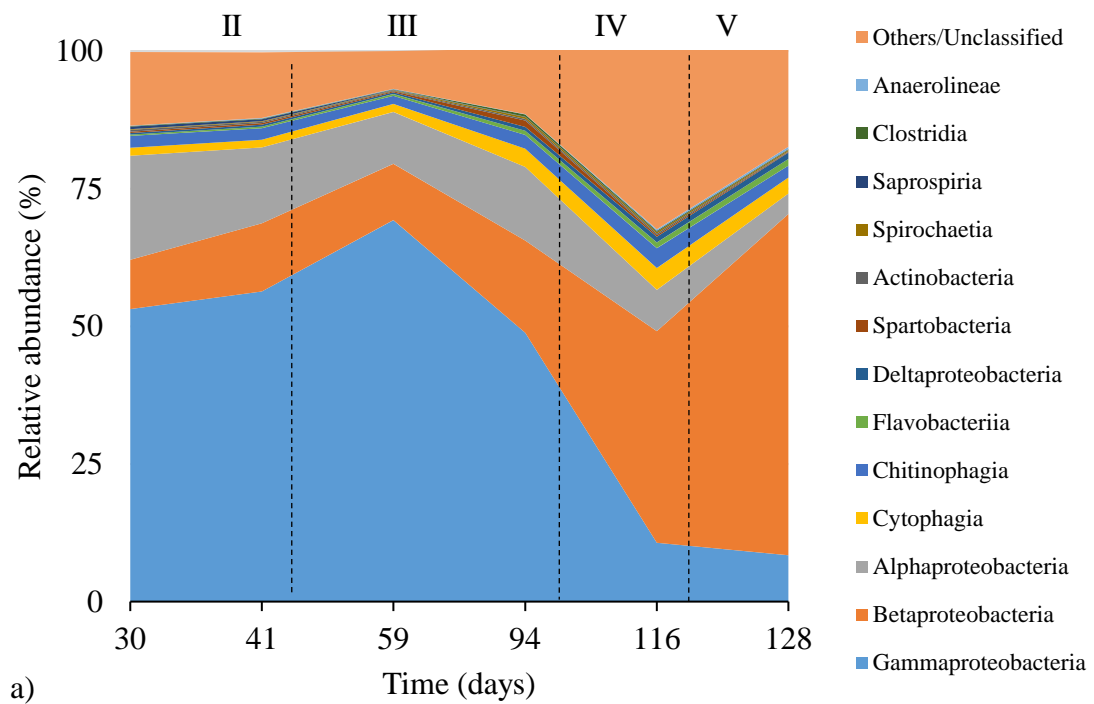


Figure 3.12 - Relative abundance of bacterial groups in class (a) and order (b) levels.

Concerning class level, there was a change in the relative abundance profile in the classes of Proteobacteria phylum over the operation time (Figure 3.12a). Gammaproteobacteria frequency increased from 53% (day 30 – before the EDCs

supplementation) to 69% (day 59 – phase III) and then decreased significantly up to 8% (day 128 – after removing EDCs from the influent). The same pattern was observed for Alphaproteobacteria, but the proportion of this class was not so significant (19% - day 30 to 4% - day 128). For Betaproteobacteria, an opposite trend was observed; there was an increase from the start to the end of reactor operation (9% to 62%), so that this class became the dominant one. Chitinophagia and Cytophagia did not show much difference in relative abundance, with an average value of 2.4% for both classes. The remainder classes, such as Flavobacteria and Deltaproteobacteria, corresponded to less than 1% of the microbial community in each sample.

Analyses at the order level were conducted to reveal more information on the microbial community structure in the samples from the AGS-SBR (Figure 3.12b). The relative proportion of *Enterobacteriales* increased from 37.4% (day 30) to 49.8% (day 59) and then decreased to 2.7% (day 116). *Rhodocyclales*-related organisms were present in the reactor on day 30 (4%), and their frequency gradually increased from day 94 (9%) until day 128 (51.9%). Conversely, the relative abundance of *Rhizobiales*, *Rhodobacteriales*, and *Shingomonadales* orders decreased throughout the operation time. Other orders, such as *Caulobacteriales* and *Saprospirales*, represented less than 0.5% of the microbial community.

The evolution of the most dominant bacterial genera in granular biomass was assessed and shown in Table 3.4. *Klebsiella* genus, within the order *Enterobacteriales*, was dominant from the beginning of reactor operation (phase I) to day 94 (phase III). Bacteria belonging to the genus *Klebsiella* were reported to be important players in antibiotic degradation by activated sludge (YANG *et al.*, 2020). The genus *Propionivibrio*, assigned to the order *Rhodobacteriales*, became the most dominant one (phase IV and V). *Propionivibrio*-related organisms were described as novel GAOs present in EBPR systems, commonly found on full-scale WWTPs, where they often co-exist with *Candidatus Accumulibacter phosphatis* (ALBERTSEN *et al.*, 2016). Even with a much higher proportion of GAOs compared to PAOs in the microbial community, especially in run V, when the proportion of the first reached 40%, high phosphate removals were obtained. This can be associated with the increased frequency (0.74 to 3.43%) of the PAOs belonging to *Dechloromonas* genus from phase I to phase V. Also affiliated to the order *Rhodobacteriales*, *Zoogloea* showed an increasing trend over time, however, with lower abundance when compared to *Propionivibrio*. Microorganisms

within the *Zoogloea* genus have been typically found in activated sludge, being responsible for the formation of sludge flocs (DONG *et al.*, 2017). Moreover, *Zoogloea*-related bacteria were found in significant amounts in pharmaceutical treatment processes, indicating their importance in the degradation of such compounds (ZHAO *et al.*, 2015). The presence of *Zoogloea* in the AGS-SBR may have contributed to the cohesion of the granules, besides playing an important role in estrogens biodegradation. It was suggested that denitrifying bacteria can also accumulate polyphosphate, and the denitrifying organisms of the genus *Pseudomonas* have shown the ability to store poly-P without synthesizing PHA (SEVIOUR *et al.*, 2003). An increment of *Pseudomonas* frequency was observed during the phases when EDCs were added to the influent (phases II and III) and decreased considerably from 16% to 1% (from day 94 to day 116). Increased abundances of *Pseudomonas* and *Luteimonas* in BPA-exposed biofilm indicate that these genera may have played an important role in BPA biodegradation (CYDZIK-KWIATKOWSKA; ZIELIŃSKA, 2018). *Pseudomonas* was found to be one of the most common genera in an AGS reactor treating wastewater containing aniline (JIANG *et al.*, 2017). *Methylophilus* was the only genus whose relative importance increased in the presence of EDCs and returned to values initially found (phase I) when the EDCs were removed from the influent (phase V). The increase in the abundance of these organisms may imply that they are possibly associated with EDCs removal, showing resistance to the toxicity of these compounds.

Table 3.4 – Heatmap showing the evolution of dominant genera in the granular biomass over the experimental phases. Only the most abundant bacterial genera were considered (20 genera more abundance).

1	Genus	I	II	III	IV	V		
		Day 30	Day 41	Day 59	Day 94	Day 116		Day 128
●	<i>Klebsiella</i>	33.21	32.11	48.83	23.59	2.48	2.21	0 - 1
●	<i>Propionivibrio</i>	2.41	2.86	1.81	5.84	17.12	40.67	1 - 2.5
●	<i>Pseudomonas</i>	7.93	15.74	13.49	16.33	1.25	1.06	2.5 - 5
●	<i>Methylophilus</i>	1.17	2.70	2.84	4.40	7.16	1.07	5 - 10
●	<i>Zoogloea</i>	0.81	1.17	0.47	0.85	3.14	6.70	10 - 20
●	<i>Dechloromonas</i>	0.74	0.81	1.32	1.65	2.45	3.43	20 - 100
●	<i>Runella</i>	1.13	1.10	1.21	2.48	1.69	1.25	
●	<i>Novosphingobium</i>	0.95	1.07	0.91	2.72	1.50	0.37	
●	<i>Gemmobacter</i>	1.98	1.49	0.90	1.37	0.70	0.30	
●	<i>Methyloversatils</i>	1.29	2.39	1.36	0.45	0.28	0.22	
●	<i>Terrimonas</i>	0.54	0.40	0.30	0.92	2.58	1.09	
●	<i>Luteimonas</i>	0.95	1.56	0.96	0.62	0.62	0.39	
●	<i>Pseudoxanthomonas</i>	1.88	0.97	0.32	0.40	0.23	0.12	
●	<i>Chelatococcus</i>	1.75	0.89	0.34	0.27	0.18	0.11	
●	<i>Thiothrix</i>	0.29	0.31	0.20	0.27	0.52	1.91	
●	<i>Sphingobium</i>	0.24	0.25	1.14	1.08	0.27	0.13	
●	<i>Fluviicola</i>	0.13	0.14	0.16	0.56	0.92	1.06	
●	<i>Sphingosinicella</i>	0.39	0.52	0.47	0.60	0.31	0.21	
●	<i>Ravibacter</i>	0.16	0.13	0.17	0.68	0.53	0.32	
●	<i>Rurimicrobium</i>	0.51	0.74	0.24	0.10	0.07	0.07	

¹ Color indicates the functional groups to which these organisms belong (see Figure 3.13).

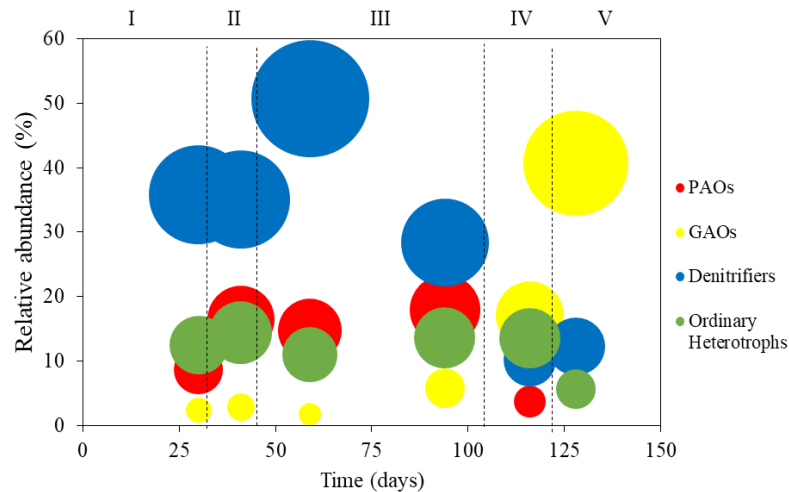


Figure 3.13 - Relative abundance (%) of the main microbial functional groups (PAOs, GAOs, denitrifiers and ordinary heterotrophs – described only as heterotrophs) within the AGS system. The genera associated with these groups are displayed in Table 3.4.

Some genera of heterotrophic bacteria, such as *Pseudoxanthomonas*, *Thiothrix*, *Sphingobium*, and *Fluviicola*, showed very low abundance (<2%) throughout the entire operating period, which may indicate that they were not so relevant for the conversion processes taking place in the aerobic granules. Furthermore, the sequencing results revealed a small proportion of AOB and NOB within the bacterial community, even though high nitrification levels were achieved. *Nitrospira*, one of the main NOB, and *Nitrosomonas*, one of the most important AOB found in granular biomass, were detected only in low relative abundance in all samples (0.01% on average). This is the reason why these organisms are not shown in Figure 3.13, despite their key role in nitrogen conversions. Nevertheless, finding nitrifiers at very low relative abundances in 16S rRNA amplicon sequencing does not necessarily imply a low nitrifying activity (ORTEGA *et al.*, 2021).

Denitrifying bacteria had the higher relative abundance among the bacterial functional groups in phases I, II and III (Figure 3.13), indicating that this group was dominant and possibly relevant for EDCs removal. GAOs, on the other hand, became more dominant in phases IV and V. The contribution of ordinary heterotrophs to the overall bacterial community was similar in all phases, indicating that this group was not affected by EDCs.

The use of indices helps to better understand the behaviour of the microbial community throughout reactor operation. Simpson index quantifies the probability of getting distinct OTUs from two consecutive random sequences. On the other hand, the Shannon diversity index (H) characterizes the diversity of bacterial communities (SHANNON; WEAVER, 1963). The results showed that the Simpson index increased after the addition of EE2 in the EDCs mixture (day 59), indicating greater dominance and less diversity, which is in agreement with the decrease of the Shannon index (Figure 3.14a). The same pattern was observed after removing EDCs from the influent (phase V), but the changes were less pronounced.

The Bray-Curtis index is a statistical method used to quantify the compositional dissimilarity (distance) between samples based on counts of the OTUs in each sample (BRAY; CURTIS, 1957). The coordination, shown in Figure 3.14b, groups the closest (most similar) samples, and the lines connect the closest samples. The sample from day 41 exhibited the most similar composition between the samples because it was closer to that of days 30, 59, and 94. Besides that, there was a clear change in the microbial community composition over time, given that the sample from day 30 (last day of phase I, without EDCs in the influent) and day 128 (last operating day) are on opposite sides of the graph. This result indicates that the microbial community changed irreversibly after EDCs addition to the reactor influent, and did not return to its initial composition found in phase I, even after the removal of EDCs.

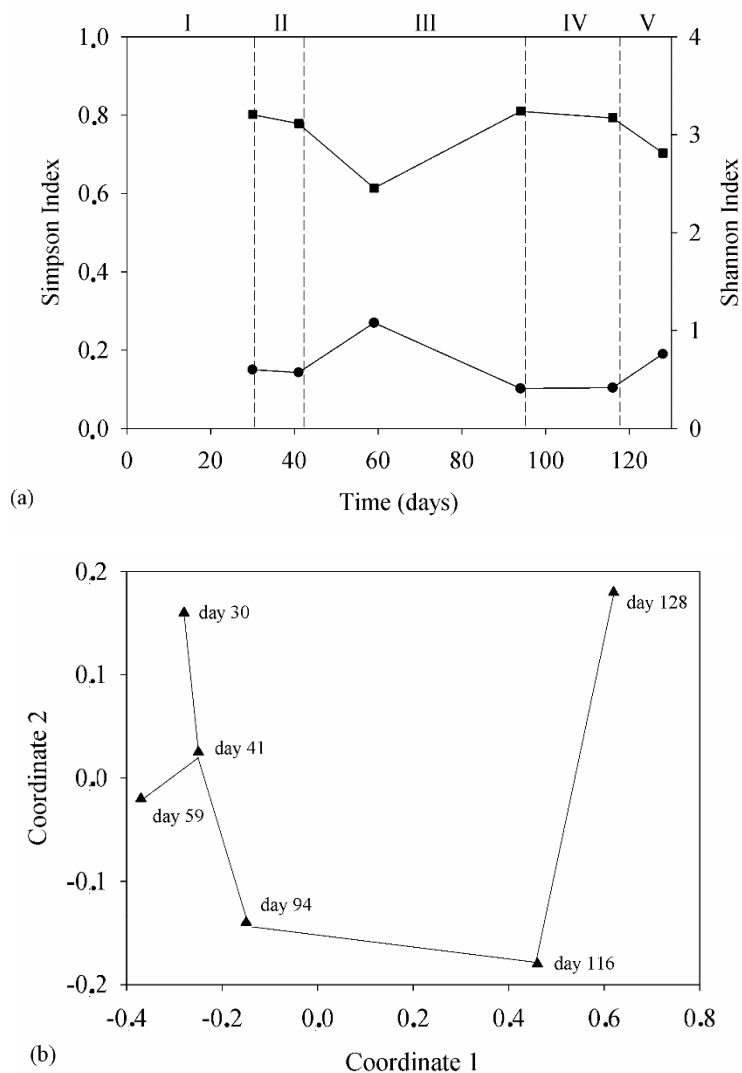


Figure 3.14 - Richness and diversity index (a) and Bray–Curtis dissimilarity (b). Simpson (●), Shannon (■) and Bray–Curtis index (▲) are represented.

3.4 Conclusions

In this study, the contribution of adsorption and biodegradation mechanisms on EDCs (E2, BPA, and EE2) removal in an AGS-SBR subjected to alternating anaerobic-aerated conditions was assessed. E2 was initially sorbed on the granular biomass in the anaerobic feeding, and then metabolized. Under aeration, the adsorbed E2 was released and fully degraded by aerobic granules. BPA was not readily metabolized, but after acclimation of the microbial community, similar behavior to that shown by E2 was observed. The adsorption under anaerobic conditions was lower for EE2 than the other compounds. In the aerated period, there was also a release of EE2 previously sorbed in

the biomass, but biodegradation was marginal. Furthermore, EE2 negatively affected phosphate removal and nitrification processes. 16S rRNA gene sequencing revealed that the microbial community significantly changed during the experiment, with a substantial increase in the abundance of microorganisms belonging to the genus *Methylophilus* after EDCs supplementation. These may have played an important role in the EDCs removal. Moreover, *Klebsiella* genus was dominant from phase I (without EDCs supplementation) until the phase III (E2, BPA and EE2 fed to the reactor), while the genus *Propionivibrio* became the most dominant one from phase III to the end of operation (phase V - withdrawal EDCs influent). Even with the reactor with a relatively high proportion of GAOs, high phosphate removals were obtained. This was likely attributed to the increased frequency of *Dechloromonas*-related PAOs, and *Pseudomonas*, a denitrifying bacteria that can also accumulate polyphosphate, found in higher proportions during the phases when EDCs were added to the influent.

4 TREATMENT OF SIMULATED SALINE WASTEWATER AMENDED WITH ENDOCRINE-DISRUPTING CHEMICALS BY AEROBIC GRANULAR SLUDGE: EVALUATING PERFORMANCE AND MICROBIAL COMMUNITY DYNAMICS

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4.1 Introduction

Endocrine-disrupting chemicals (EDCs) are a kind of emerging contaminants that can disturb the normal hormone activities of humans and animals, causing adverse effects on their behaviour, growth, and immune function (DEBLONDE *et al.*, 2015; LUO *et al.*, 2014). Pharmaceuticals and personal care products (PPCPs), hormones, industrial chemicals, surfactants, pesticides are some of the emerging contaminants (CHANG, H. *et al.*, 2009).

Among the hormones commonly found in environmental matrices, 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2) stand out (ADEEL *et al.*, 2017; NAZARI; SUJA, 2016). E2 is a natural estrogen crucial for the development and maintenance of female reproductive organs (BRUNTON *et al.*, 2005). Livestock is one of the main sources of environmental contamination by E2 since this natural hormone is excreted by animals (SCHUH *et al.*, 2011). The excretion of E2 derived from pharmaceutical uses, including hormone replacement and treatment of some cancers, contributes only 5% to the overall contamination by this compound (de MES *et al.*, 2005). The exposure of aquatic organisms to E2 causes damage and health-related conditions (NAZARI; SUJA, 2016). On the other hand, EE2 is a synthetic estrogen produced from the natural hormone E2. It is the main ingredient in most contraceptives and has other applications, such as interruption of breast milk production and hormone substitution (ARIS *et al.*, 2014). EE2 has adverse ecological effects at concentrations lower than 4 ng L⁻¹ (KIDD *et al.*, 2014).

The clearest sign has been observed in male fish exposed to estrogen (EE2-E2) impacted water bodies at 1–2 ng L⁻¹, presenting male and female sexual characteristics, such as partial evolution of eggs or ova (WOODS; KUMAR, A., 2011).

Besides the natural and synthetic hormones, another important contaminant of water resources is the xenoestrogen bisphenol-A (BPA). Largely produced worldwide (about three million tons per year), its main use is to manufacture plastic and epoxy resins (LE CORRE *et al.*, 2015). Even though BPA exhibits low estrogenic activity (10,000 to 100,000 times weaker than that of E2) (WELSHONS *et al.*, 2006), it has been associated with serious health effects in humans and wildlife, causing several endocrine disorders (SHAFEI *et al.*, 2018).

E2, EE2 and BPA reach the environment due to inadequate disposal of non or partially treated domestic or industrial wastewater, the latter commonly associated with the inefficiency of wastewater treatment plants (WWTPs) (BOSHIR *et al.*, 2017; DEBLONDE *et al.*, 2015; KOH *et al.*, 2008). For example, the conventional activated sludge (CAS) and membrane bioreactor (MBR) processes commonly implemented in WWTP appear to be unable to biodegrade these compounds completely, as some of them are toxic to microorganisms and resistant to microbial degradation (BOSHIR *et al.*, 2017; GARCIA-BECERRA; ORTIZ, 2018). Aerobic granular sludge (AGS) has been seen as a breakthrough in biological treatment, showing several properties that make it more attractive than CAS-based processes: higher compactness, capability of simultaneous removal of carbonaceous matter and nutrients in a single reactor, high shock load resistance, and excellent settling properties (BASSIN, 2018; BEUN *et al.*, 1999; LIU; TAY, 2004). The latter is of utmost importance for allowing a good effluent clarification and high biomass retention within the bioreactor, characteristics attributed to the formation of regular and dense self-immobilized microbial clusters (ADAV *et al.*, 2008; BEUN *et al.*, 1999). Such structure enables the development of many functional microbial groups acting in the biodegradation of refractory organic compounds (CAI, F. *et al.*, 2021). Furthermore, the granular biomass layered structure leads to concentration gradients within the granule, protecting microorganisms from the toxicity associated with wastewater compounds (MASZENAN *et al.*, 2011). AGS has also been shown to be a suitable biological process for the degradation of emerging contaminants of diverse nature (AMORIM *et al.*, 2014, 2016; BALEST *et al.*, 2008; CYDZIK-KWIATKOWSKA

et al., 2017; KENT; TAY, 2019; LI *et al.*, 2015; MOREIRA *et al.*, 2015; WANG; IVANOV, 2009).

Despite these key attractive features, the same challenges experienced in CAS reactors are also faced in AGS systems, especially concerning the removal of some specific micropollutants. Bioaugmentation, through the addition of specific single strains or microbial consortia, can enhance the biodegradation of target micropollutants (BENNER *et al.*, 2013), and has been used in AGS systems for treating, for example, herbicides (QUAN *et al.*, 2015) and organofluorines (DUQUE *et al.*, 2011; OLIVEIRA, A. S. *et al.*, 2021). However, most previous research addressing emerging contaminants removal by AGS were carried out in freshwater, while few studies on this topic were conducted under high salinity conditions (RÓZALSKA *et al.*, 2015; ZHOU; LI; LEUNG, 2019). Salinity is especially important in coastal cities where seawater is used as an alternative water source for toilet flushing (WU *et al.*, 2016; WU *et al.*, 2008). As the microbial communities are often incapable of tolerating high osmotic pressures (WANG *et al.*, 2017), the efficiency of the biological process may deteriorate when the wastewater treatment facilities receive saline wastewaters (LI *et al.*, 2010).

The objective of this study was to assess the ability of AGS to remove some emerging contaminants commonly detected in domestic wastewaters (i.e., E2, EE2 and BPA) under a saline environment (12 g L⁻¹ NaCl). We hypothesised that bioaugmentation with a specific EDCs-degrading bacterial strain could improve the removal of such compounds and that the AGS could be set as an enrichment reactor for degrading strains. The contribution of adsorption and biodegradation mechanisms on the removal of the target compounds and their effects on reactor performance and AGS health were also addressed. The composition of the microbial community was assessed throughout the operation.

4.2 Materials and methods

4.2.1 Chemicals

E2, EE2, and BPA standards were acquired from Sigma Aldrich (purity > 98%) (Steinheim, Germany). HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany), while a Milli-Q water purification system provided ultra-

pure water. Stock solutions of the EDCs mixtures were prepared at a concentration of 2,000 and 26,500 mg L⁻¹ in methanol and stored at -20 °C in amber glass bottles. Analytical grade chemicals (Merck, Darmstadt, Germany; Sigma–Aldrich Chemie, Steinheim, Germany) were used to prepare the SBR influent synthetic wastewater (De KREUK *et al.*, 2005).

4.2.2 AGS-SBR system

The experiment was conducted using a lab-scale AGS-SBR with 110 cm of total height, 6.5 cm of internal diameter and 2.5 L of a working volume. The system was operated in 3, 6, 8 and 12 h cycles. Automatic timers were employed to start and stop pumps during the reactor cycle. The cycles were divided into four consecutive phases: anaerobic feeding (60 min), when 0.95 L of influent was pumped into the reactor; aeration (112, 292, 412 and 645 min for the 3, 6, 8 and 12 h-cycles, respectively), during which compressed air was sparged into the reactor bottom at an airflow rate of 4 L min⁻¹; biomass settling (3 and 10 min) and effluent withdrawal (5 min). The reactor was operated at a volume exchange ratio of 39%. There was no control of the sludge retention time (SRT) throughout reactor operation, therefore, it was governed by natural suspended solids washout. The experiment was carried out with no oxygen and pH control. Nevertheless, their values remained approximately constant at 7.5 and 6 mg L⁻¹ for pH and DO, respectively. The bioreactor was operated at room temperature (25 ± 2° C). The AGS biomass used as inoculum was taken from another lab-scale reactor that had been previously exposed to relatively high salinity (14 g L⁻¹ NaCl). During the experiment, the salinity level was maintained under similar conditions.

4.2.3 Wastewater composition and operating conditions

The SBR influent wastewater was composed of two different solutions (A and B), as described by De KREUNK *et al.* (2005). Solution A comprised NaCH₃COO 3H₂O (73.5 mM), MgSO₄ 7H₂O (3.6 mM) and KCl (4.7 mM), while solution B was composed of NH₄Cl (35.5 mM), Na₂HPO₄ (4.2 mM), KH₂PO₄ (2.2 mM) and NaCl (1 M). A trace element solution (VISHNIAC; SANTER, 1957) was added to solution B (5 mL L⁻¹) to favour the microorganisms growth. In each cycle, a volume of 89 mL of solution A and

B was combined with 772 mL of water to achieve influent organic matter, ammonium and phosphate concentrations close to 450 mg L⁻¹ (as COD), 50 mg NH₄⁺-N L⁻¹, 16 mg PO₄³⁻-P L⁻¹, respectively, a typical composition of domestic wastewater (METCALF; EDDY, 1991). Small deviations from the desired values resulted from the influent preparation method and the mixing of solutions A and B by peristaltic pumps.

The bioreactor operation was divided into twelve experimental phases (Table 4.1). During phase I, acetate was the only carbon source present in the influent stream. From phase II (starting on the 14th day of reactor operation), a mixture of E2, EE2 and BPA was fed to the AGS-SBR. The mixture of EDCs was added to the influent during one cycle per day at the same cycle of the day. An appropriate quantity of the stock solution containing these compounds (2,000 mg L⁻¹ of E2, EE2 and BPA, dissolved in methanol (phase II)) was added to solution A to reach an influent concentration of 2 mg L⁻¹ of each compound. This concentration was set because of analytical limitations to detect and quantify them at the concentrations in which usually occur in environmental matrices, but above all, to allow a better investigation of removal mechanisms. In phase IV, a stock solution containing 26,500 mg L⁻¹ of EDCs was prepared with the purpose of using a smaller amount of methanol, thus reducing its contribution to the influent COD. After this phase, the reactor had to be deactivated, and therefore, the biomass was stored under refrigeration at 4 °C for 60 days (this period has not been counted for the SBR operation and occurred during the covid lockdown). The biomass was then reactivated for 17 days without the presence of EDCs (phase V).

For bioaugmentation of the SBR (phase VII) intended to improve the micropollutants removal efficiency, a previously isolated specialized strain of *Rhodococcus* sp. ED55, reported to be able to degrade EDCs (MOREIRA, I. S. *et al.*, 2021), was used. ED55 pure cultures were placed in sealed flasks with a mineral salts medium and were incubated in an orbital shaker at a temperature of 25 °C and a rotating speed of 100 rpm. The optical density at 600 nm (OD600) was measured for microbial growth monitoring. The reactor was inoculated, respectively, with 20 mL and 11 mL of an ED55 pure culture with an OD600 of 0.614 and 0.810 after 20 and 28 min that the aerating period was started. During this period, the cycle time was increased 12 h, so the aeration and settling stages were extended to 652 min and 10 min, respectively. The duration of the other phases of the SBR remained invariant. This operating strategy was

intended to maximize the permanence time of the strain within the reactor, preventing it from being washout with the effluent from the reactor.

During phase XI, the ammoniacal load was increased from 0.19 to 0.37 g m⁻³ d⁻¹. In the last regime (phase XII), the reactor returned to the initial imposed conditions (EDCs-free condition). The variation of influent COD and EDCs loads over the reactor operation associated with cycle time changes are displayed in Table 4.1.

Table 4.1 - Summary of the operating conditions tested in the SBR.

Phase	SBR operating conditions									
	Operation length (days)	Cycle time (h)	Influent feeding (min)	Aeration (min)	Settling (min)	Effluent withdrawal (min)	HRT (h)	InfluentCOD load (kg m ⁻³ d ⁻¹)	Influent load (mg m ⁻³ d ⁻¹)	EDCs
I	1 -13	3	60	112	3	5	7.7	1.0	0	
II	14 - 37	3	60	112	3	5	7.7	1.4	18.6	
III	38 – 49	3	60	112	3	5	7.7	1.0	0	
IV	50 – 59	3	60	112	3	5	7.7	1.0	18.6	
V	60 – 77	3	60	112	3	5	7.7	1.0	0	
VI	78 – 83	3	60	112	3	5	7.7	1.0	18.6	
VII	84 – 92	12	60	645	10	5	30.9	0.3	4.6	
VIII	93 – 99	8	60	412	3	5	20.6	0.4	7.0	
IX	100 – 113	6	60	292	3	5	15.5	0.5	9.3	
X	114 – 120	3	60	112	3	5	7.7	1.0	18.6	
XI	121 - 132	3	60	112	3	5	7.7	1.0	18.6	
XII	133 - 140	3	60	112	3	5	7.7	1.0	0	

4.2.4 Analytical methods

Samples routinely collected at the bioreactor influent stream, after the anaerobic feeding phase, and effluent were subjected to filtration in nylon membrane syringe filters (0.45 mm pore size) for biomass removal. EDCs (E2, EE2 and BPA) were analyzed using a reversed-phase 250e4 HPLC-Cartridge LiChrospher 100 RP-18 column (Merck). The mobile phase comprised eluent A (water acidified at pH 2.2 with trifluoroacetic acid) and eluent B (acetonitrile). The elution started with 40% of eluent B for 4 min, followed by a gradient of 60% of eluent B (4 min) and 40% of eluent B (6 min), totalizing a run of 14 min at a constant flow rate of 0.8 mL min⁻¹. E2, EE2, and BPA retention time were 11.6, 12.1, and 10.0 min, respectively.

Spectroquant[®] (Merck Millipore) photometric test kits were used to measure COD, phosphate, ammonium, nitrite and nitrate concentrations in filtered samples, following the manufacturer's recommendations. Total and volatile suspended solids (TSS and VSS, respectively) were quantified following Standard Methods (APHA, 2005). The AGS bed height was determined at the beginning of the feeding period through a ruler positioned over the reactor column.

The amount of incorporated ammonium nitrogen (gN d⁻¹) for cell growth was estimated according to the modified equation (WAN, J.; BESSIÈRE; SPÉRANDIO, 2009):

$$N_{\text{assimilated}} = f_N \times VSS_{\text{effluent}} \times O \quad (4.1)$$

Where f_N is the fraction of nitrogen in the sludge (0.12389 mgN mgVSS⁻¹); O is the output (L d⁻¹); VSS_{effluent} is the concentration of solids in the final effluent (gVSS L⁻¹).

Quantitative image analysis (QIA) was employed to observe morphological and physical characteristics of granules. Duplicate AGS biomass samples were collected during the middle of the aeration period over the reactor operation, washed with phosphate-buffered saline (PBS) solution, and incubated in a mixture of PBS and formaldehyde 4% (0.25:1) for 2 h at 4 °C. Then, the biomass was washed with PBS and preserved in a solution of PBS and ethanol 96% (1:1). The granules were transferred to a Petri dish for visualization and image acquisition through an Olympus magnifying glass, with a total magnification of 15x. The granules were classified into two classes based on their equivalent diameter (D_{eq}): IG – Intermediate-sized granules (0.15 mm < D_{eq} < 1.5

mm), and LG – Large-sized granules ($D_{eq} \geq 1.5$ mm). The acquired images were processed using the Matlab program (The Mathworks, Inc., Natick), as described by RAMOS *et al.* (2017).

The extracellular polymeric substances (EPS) content was analyzed upon their extraction by a heat method, according to FELZ *et al.* (2016). The carbohydrate content in EPS was estimated by the anthrone-sulfuric acid method, using glucose as standard (FROLUND *et al.*, 1996), while protein and humic acids contents were estimated by a modified Lowry method with bovine serum albumin as standard (FROLUND *et al.*, 1995).

4.2.5 Isolation and identification of EDCs degrading strains from the SBR

Isolation of possible EDCs degrading strains was carried out by plating serial dilutions of crushed granules onto agar (LABM, UK), to which the SBR synthetic influent medium and 5 mg L⁻¹ of each selected compound (E2, EE2, and BPA) were added. A volume of 100 µL of each dilution was spread onto the plates and incubated for 3 days at 25 °C. The bacterial colonies were isolated by means of the streak–plate procedure, taking into account the size, morphology, and pigmentation. After the growth of the isolated strains, they were incubated on an orbital shaker at 25 °C and 100 rpm to evaluate their E2, EE2, and BPA degradation capacity. Two 250 mL flasks containing 50 mL of SBR synthetic influent with 5 mg L⁻¹ of each compound were tested. DNA extraction and sequencing analysis of the EDCs-degrading strains followed the description made by DUQUE *et al.* (2011).

4.2.6 Microbial community analysis of AGS

4.2.6.1 DNA extraction

AGS samples were withdrawn from the bioreactor during the middle of aeration period at the end of some experimental phases. The genomic DNA extraction of aseptically crushed AGS samples was performed using the UltraClean Microbial DNA Isolation Kit (MoBio, USA), based on the manufacturer's protocol. The extracted DNA was kept at -20 °C until it was used for analysis.

4.2.6.2 Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA

To amplify the variable V3 region of bacterial 16S rRNA gene fragments, primers 338F-GC and 518R were used (MUYZER *et al.*, 1993). The PCR reaction mixture and temperature profile were set as described by AMORIM *et al.* (2014). The reactions were performed in a Bio-Rad iCycler (Bio-Rad, USA). A DCode Universal Mutation Detection System (Bio-Rad, USA) (35% to 70% of denaturing gradient, with 100% denaturant defined as 7 M urea and 40% formamide) was used to separate PCR-amplified 16S rRNA gene fragments by DDGE. Electrophoresis was conducted in 1x TAE buffer at 60 °C and 20 V (the first 15 min) and 75 V (960 min). A 10x GelGreen Nucleic Acid Stain solution (Biotium Inc., USA) at 0.1 M NaCl concentration was used to stain the gels.

DGGE profiles were analysed employing Bionumerics software (Applied Maths, Belgium). Based on Pearson similarity coefficient with 1% tolerance, a dendrogram was elaborated and clustered with the unweighted pair group mean average (UPGMA) method. Shannon diversity index (H) (SHANNON; WEAVER, 1963) and equitability index (E) (PIELOU, 1975) were used to assess bacterial diversity and evenness of the microbial community, respectively.

4.2.6.3 Sequencing of DNA from DGGE bands and bioreactor biomass samples

A sterile scalpel was used to excise selected DGGE bands and eluted in 50 µL of sterile 10 mM Tris-HCl buffer (pH 8.00). After 48 h of incubation at 4 °C, the supernatant was re-amplified with the original primer set, which did not contain the GC clamp on the forward primer (338F). Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), were used to purify PCR products. For identification and phylogenetic classification, band sequences were matched with the BLAST software from National Centre of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>).

Next-generation sequencing (NGS) was performed using DNA from bioreactor biomass samples, without replicates. GATC-Eurofins (Konstanz, Germany) were used for all procedures, including amplification of DNA, preparation of libraries, sequencing and bioinformatic analysis. Two primers, covering the V3-V4 hypervariable region,

(357F - TACGGGAGGCAGCAG (TURNER *et al.*, 1999); 800R - CCAGGGTATCTAATCC (KISAND *et al.*, 2002)) were used, and paired end sequencing based on 16S rRNA gene was conducted (Illumina MiSeq platform). Demultiplexing, clipping of primer sequences, merging, quality filtering and microbiome profiling integrated the steps of the microbiome analysis pipeline. To assign taxonomic information of the OTUs, DC-MEGABLAST alignments of cluster representative sequences were performed. Reference sequences with a minimum of 70% and 80% of identity and representative sequence, respectively, were selected and then processed with the QIIME software package (version 1.9.1, <http://qiime.org/>). The raw sequence data were deposited in Sequence Read Archive (SRA) from NCBI database, associated with the BioProject, under the accession number PRJNA645158.

4.3 Results and discussion

4.3.1 EDCs removal

Figure. 4.1 shows the average mass of each compound during the cycle subjected to shock load in the influent, after the anaerobic feeding phase, and effluent, while the concentrations throughout the operating days are presented in Figure. 4.2.

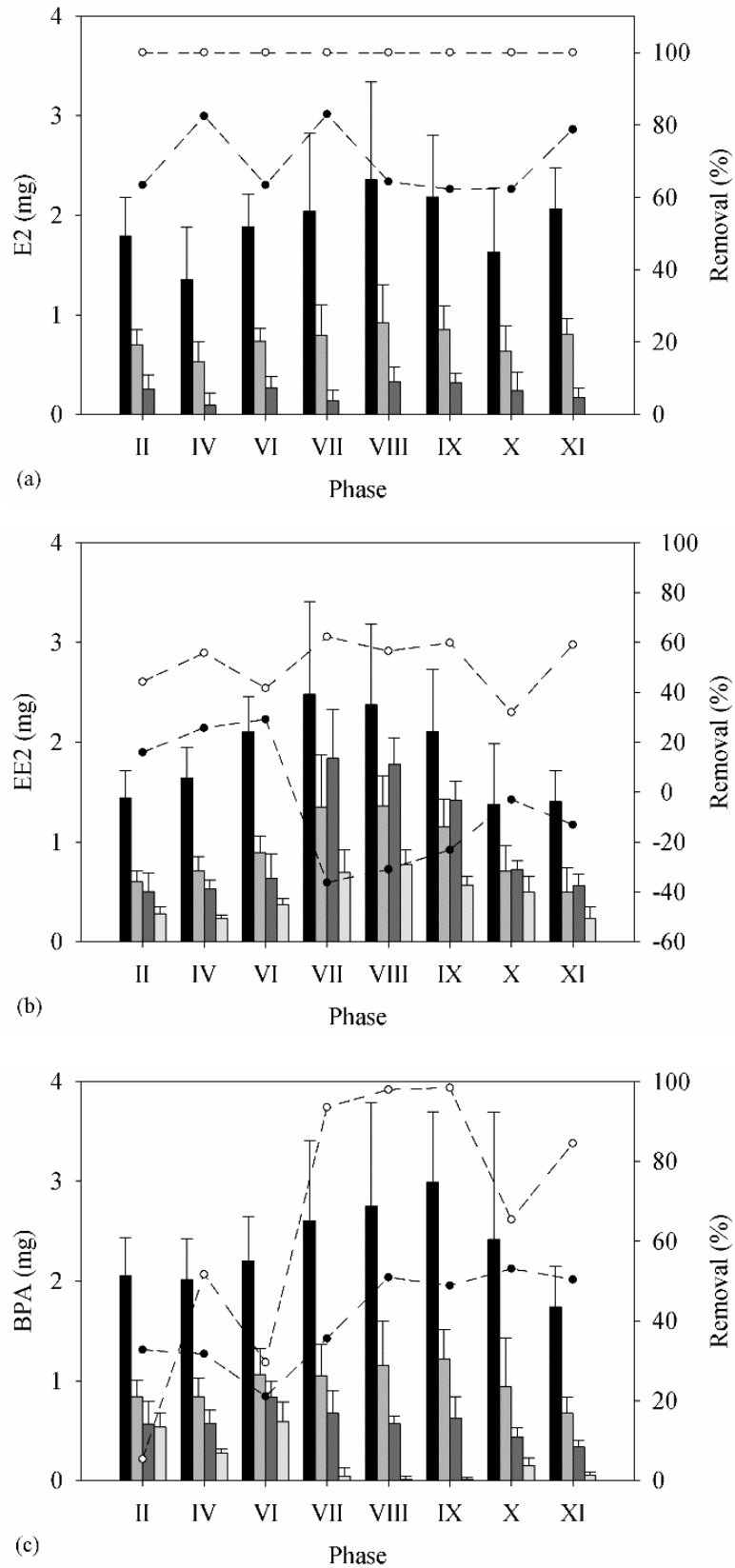


Figure 4.1 - Average mass of the E2 (a), EE2 (b) and BPA (c) during the cycle subjected to EDCs load. Legend: Influent (black bar), after anaerobic feeding – theoretical (medium

grey bar) and measured mass (dark grey bar), effluent (light grey bar), anaerobic removal (●) and removal under aeration (○).

In each cycle, the anaerobic period responded for the removal of 60% to 80% of E2, while the rest was quickly consumed in the aerated phase, as shown in the data from the cycle tests (Figure. 4.3). Biodegradation was assumed as the governing removal mechanism as the compound was removed from the bulk liquid and was never detected in the effluent. As reported in previous investigations, E2 exhibits high biodegradability. Fresh conventional activated sludge (CAS) rapidly oxidized E2 under aerobic conditions at concentrations of 1 mg L^{-1} and $1 \text{ } \mu\text{g L}^{-1}$ after 1 and 3 h, respectively (TERNES *et al.*, 1999), and a concentration of 5 to $15 \text{ } \mu\text{g L}^{-1}$ was completely biodegraded in 2 h (ZENG, Q. *et al.*, 2009). KASSOTAKI *et al.* (2019) observed that E2 at $15 \text{ } \mu\text{g L}^{-1}$ was always fully removed regardless of the conditions applied, whether by an enriched nitrifying activated sludge (NAS) and enriched ammonia-oxidizing bacteria (AOB) culture or by a CAS mixed bacterial consortia.

EE2 was initially removed in the anaerobic period within the shock period. This can be inferred because the EE2 concentration after anaerobic feeding was lower than expected based on its influent concentration and dilution in the reactor with the liquid remaining from the previous cycle (Figure 4.1b). This removal was most probably attributed to EE2 adsorption onto the aerobic granules. However, this trend changed after phase VII, as the concentration after feeding was higher than that expected, implying a negative net removal under anaerobic conditions. This result is probably associated with the release of EE2 adsorbed in the granular biomass to the bulk liquid during the anaerobic feeding period. Indeed, results from the cycle tests (Figure 4.4) shows that EE2 concentration increased at the beginning of the aerated stage. The analyses performed in the subsequent cycles also suggest the desorption of EE2 from the granular biomass to the liquid because even after stopping the shock loads, EE2 was still detected in the effluent (Figure 4.3a).

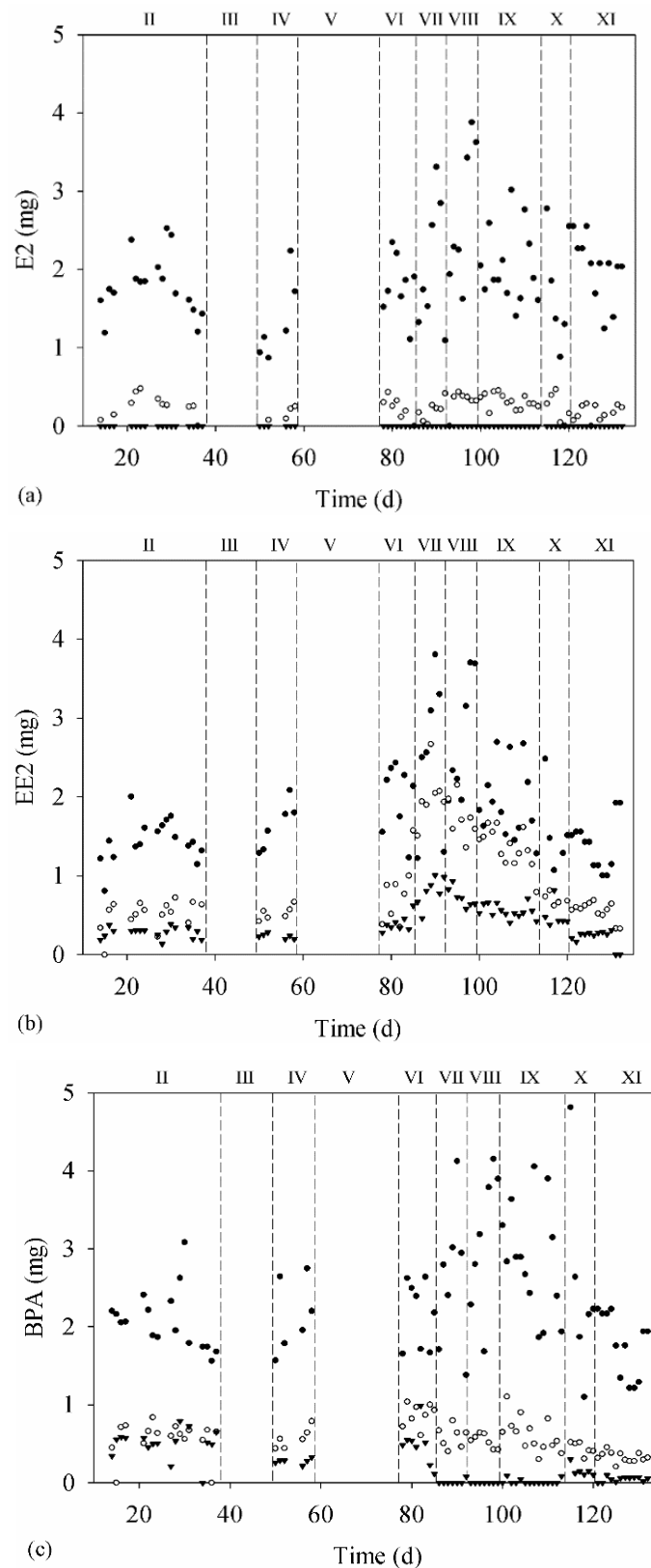


Figure 4.2 - EDCs concentration in the cycle with the shock load: (a) E2, (b) EE2, and (c) BPA. Symbols: (●) influent, (○) after anaerobic period, (▼) effluent concentration. These compounds were not added to the influent stream in phases III and V.

The total removal of EE2 became higher in phase VII (47%), when the reactor was bioaugmented with the specialized strain of *Rhodococcus* sp. ED55, and the HRT was set to 30.9 h. However, the removal dropped to 5.9% in phase VIII, even though the HRT was considerably higher (20.6 h) when compared to the initial phases (7.7 h). In phases X and XI, a negative EE2 removal efficiency was observed, implying that this compound was not removed from the system. A similar result was found by MOREIRA *et al.* (2015) for fluoxetine, which was sorbed onto the biomass until its sorption capacity became exhausted, after which desorption occurred, the latter being more evident in the periods of absence of fluoxetine. KENT; TAY (2019) studied the removal of a mixture of EE2, 4-nonylphenol, and carbamazepine (all at a concentration of 0.5 mg L⁻¹) from synthetic wastewater using AGS. Unlike what was observed in this research, the authors concluded that when the adsorption capacity saturated and biodegradation became the prevailing removal pathway, achieving removal efficiency of EE2 stabilized at an average of 77%. Also working with an AGS-SBR, CASTELLANOS *et al.*, (2021) reported removal of 99% for E2 and 93% for EE2, both fed to the reactor at a concentration of 20 µg L⁻¹. In their study, E2 and EE2 profiles over the SBR cycle suggested a rapid initial adsorption of these compounds onto the granular biomass occurring anaerobically, followed by biodegradation under aeration. MAURÍCIO *et al.* (2018) used synthetic and real wastewaters to examine the removal of 100 µg L⁻¹ of E2 and EE2 in a rotating biological contactor (tertiary treatment stage). All assays showed removal efficiency above 50% for E2. The highest removals were observed for real wastewater. More than 15% reduction of EE2 concentrations was noticed in the tests using synthetic wastewater. Nevertheless, when real wastewater was employed, no removal was achieved. These authors also observed that EE2 was more hardly removed compared to E2. The recalcitrant behaviour of EE2 was expected since it is a synthetic hormone, proceeding from E2, with an ethynyl group at the C-17 position. The latter blocks access to the hydroxyl group situated in the same position, making the compound more difficult to be biodegraded (CLOUZOT *et al.*, 2008).

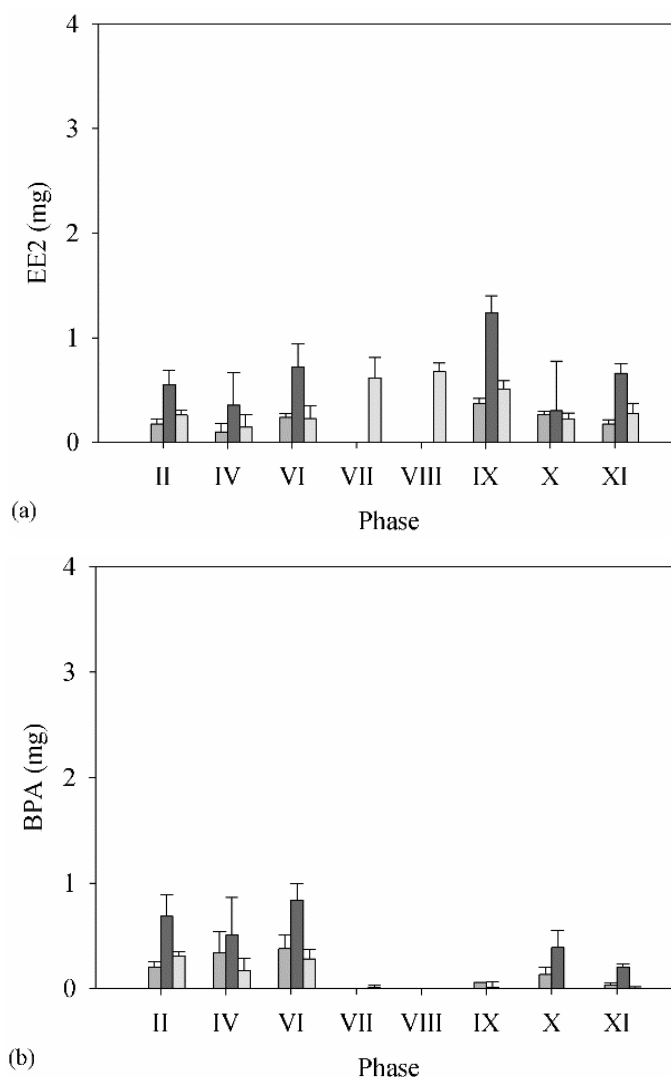


Figure 4.3 - Average mass of EE2 (a) and BPA (b) during the first cycle without EDCs. Legend: After anaerobic feeding – theoretical (medium grey bar) and measured mass (dark grey bar), effluent (light grey bar).

Removal of BPA occurred in the anaerobic feeding and aerated period, starting from 33% and 5% during phase II, respectively. BPA biodegradation within the aerated period gradually improved, as indicated by the increase in removal to 51% at phase IV. Anaerobic removal increased around by 30% after bioaugmentation (phase VI), remaining stable until the end of the monitoring period. No BPA was detected in the effluent in phases with longer cycle times (12, 8, and 6 h). However, when the cycle time was reduced again to 3 h (phase X), BPA started again to be detected in effluent samples, but at lower concentrations compared to the phases before the bioaugmentation. During phase XI, the overall removal increased to 95% (Table 4.2). The increasing BPA removal

efficiency upon bioaugmentation suggests that biodegradation was the governing removal mechanism. This fact is more evident when phases II and X are compared, both with the operating cycle lasting 3 h (Fig 4.4). FERNANDEZ *et al.* (2009) reported that 52–100% of BPA was removed from sewage in an activated sludge process, while no adsorption or accumulation was found in the sludge. Moreover, adsorption of BPA on the biomass contributed only to 2–3% of the overall BPA abatement from domestic wastewater, with the remaining attributed to degradation (ZHOU *et al.*, 2019).

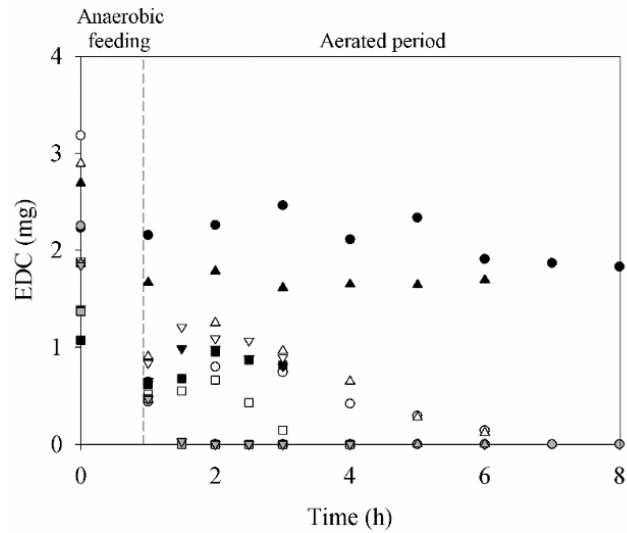


Figure 4.4 - EDCs mass profiles during a typical shock load cycle: E2 (grey), EE2 (black), and BPA (white). Symbols: Phases II – 3 h-cycle (■), VIII – 8 h-cycle (●), IX – 6 h-cycle (▲) and X – 3 h-cycle (▼).

Table 4.2 - EDCs mass balance during the shock load cycles. The mass of each compound in the effluent considers all daily cycles within a given phase.

Phase	Compound	Influent (mg d ⁻¹)	Effluent (mg d ⁻¹)	Removal (%)
II	E2	1.8 ± 0.4	<0.1	100
	EE2	1.4 ± 0.3	1.3 ± 0.4	12.5
	BPA	2.0 ± 0.4	1.2 ± 0.6	41.0
IV	E2	1.3 ± 0.5	<0.1	100
	EE2	1.6 ± 0.3	1.1 ± 0.3	32.9
	BPA	2.0 ± 0.4	1.1 ± 0.5	45.7
VI	E2	1.9 ± 0.3	<0.1	100
	EE2	2.1 ± 0.4	1.3 ± 0.3	35.7
	BPA	2.2 ± 0.4	1.9 ± 0.2	14.1
VII	E2	2.0 ± 0.8	<0.1	100
	EE2	2.5 ± 0.9	1.3 ± 0.4	47.2
	BPA	2.6 ± 0.8	0.1 ± 0.5	96.9
VIII	E2	2.4 ± 1.0	<0.1	100
	EE2	2.4 ± 0.8	2.2 ± 0.4	5.9
	BPA	2.7 ± 1.0	0.01 ± 0.03	99.6
IX	E2	2.2 ± 0.6	<0.1	100
	EE2	2.1 ± 0.6	2.0 ± 0.3	5.2
	BPA	3.0 ± 0.7	0.01 ± 0.03	99.7
X	E2	1.6 ± 0.6	<0.1	100
	EE2	1.4 ± 0.6	1.4 ± 0.1	-4.3
	BPA	2.1 ± 1.3	0.1 ± 0.1	93.2
XI	E2	2.1 ± 0.4	<0.1	100
	EE2	1.4 ± 0.3	1.6 ± 0.4	-12.8
	BPA	1.7 ± 0.4	0.1 ± 0.05	95.4

HUANG *et al.* (2019) studied the sorption behaviour of targeted EDCs, including EE2 and BPA, onto activated sludge. Rapid sorption was observed in the first 15 min, within which 79.3% (for BPA) and 84.2% (for EE2) of the total sludge sorption capacity had been reached. The equilibrium was achieved after 5 h, at which 60.9% and 49.4% of EE2 and BPA were removed from the liquid phase. However, these compounds showed

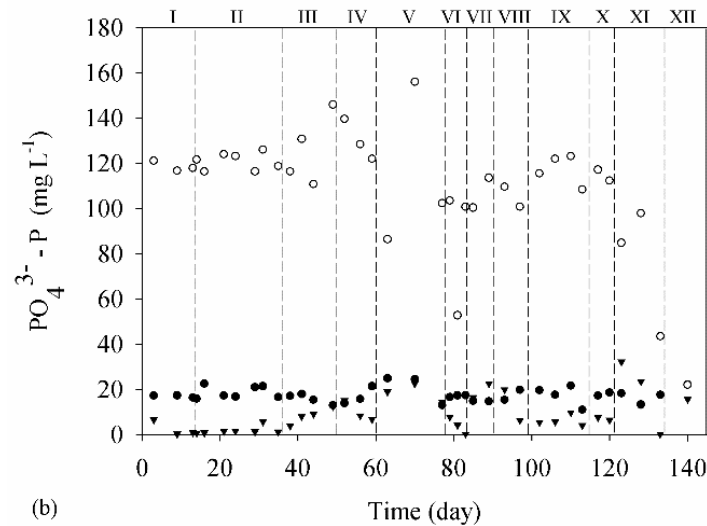
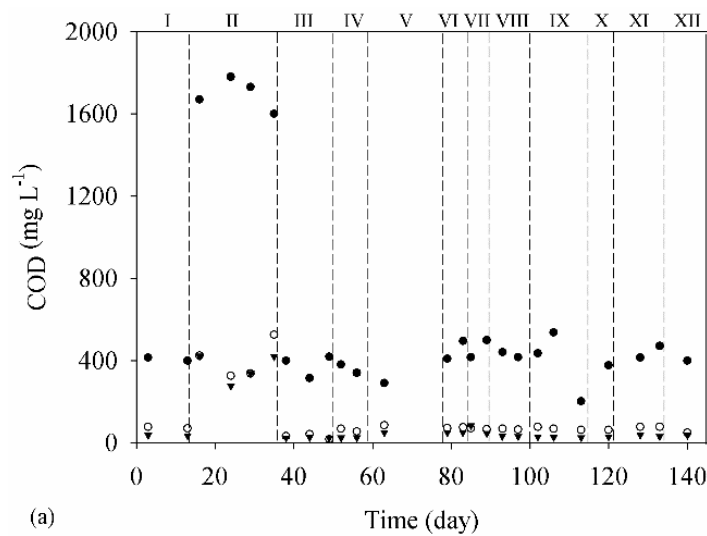
a high desorption potential, as 30–35% of the sorbed amount was dissolved back into the newly introduced wastewater.

Until now, there are still few reports on how saline wastewater interferes with the biological removal of EDCs. In particular, no previous work has addressed the removal of EDCs by AGS in saline wastewaters. The sorption of many organic pollutants onto suspended particles or sediments usually increases at higher salt concentrations due to the solubility of hydrophobic compounds that inversely depends on the dissolved salt concentration. This phenomenon is referred to as “salting-out effect” (SHI *et al.*, 2014). As a result of this effect, high salinity led to the transfer EDCs from the solution to particles and sediments in an estuary located in China (YANG *et al.*, 2016). The increase in salt concentration also allowed E2 adsorption on granular activated carbon, chitin, chitosan, ion exchange resin, and a carbonaceous adsorbent (ZHANG; ZHOU, 2005). Nevertheless, high salt concentration does not always favour adsorption. As observed by (XU *et al.*, 2008), BPA sorption on sediment increased with the salinity decrease.

4.3.2 COD, P and N removal

Within phase I, the AGS-SBR exhibited a stable COD removal efficiency, reaching effluent COD values of about 35 mg L⁻¹ (Figure 4.5a). Within the anaerobic feeding, almost all influent COD was consumed. In phase II, the EDCs mixture was incorporated into the influent wastewater, and, along with acetate, consisted of the feeding carbon sources. Methanol was used as a solvent for the EDCs stock solution containing 2,000 mg L⁻¹ of these compounds. Under these conditions, a greater amount of methanol was fed to the reactor along with the EDCs and acetate, which increased the COD loading rate, reaching 1.4 kg m⁻³ d⁻¹ (Table 4.1). Under anaerobiosis, i.e., in the absence of oxygen as a terminal electron acceptor, the selection of PAOs and GAOs is favoured (De KREUK; Van LOOSDRECHT, 2004). These organisms can intracellularly accumulate storage polymers from volatile fatty acids, e.g., acetate, used as an energy source in the aerobic phase (LOPEZ-VAZQUEZ *et al.*, 2009). With the start of phase II, an appreciable amount of external substrate (acetate and methanol) became available in the aerated phase of the cycle, allowing its use by the fast-growing heterotrophic bacteria. This fact may have decreased the relative amount of PAOs and GAOs, and consequently, COD content in the bulk liquid after the anaerobic feeding and in the effluent increased up to 430 and

360 mg L⁻¹, respectively. Then, the EDCs were removed from the influent stream at phase III. In phase IV, the EDCs mixture returned to the reactor, but the influent COD load was reduced to 1.0 kg m⁻³ d⁻¹. This was possible through the preparation of a more concentrated EDCs stock solution (26,500 mg L⁻¹), thus minimizing the interference of methanol on the incoming COD. Under these conditions, almost full COD abatement was observed during the anaerobic feeding period. The remaining COD in the effluent was due to the presence of non-biodegradable organic matter, partially present in the influent stream and partially derived from the by-products resulting from microbial degradation.



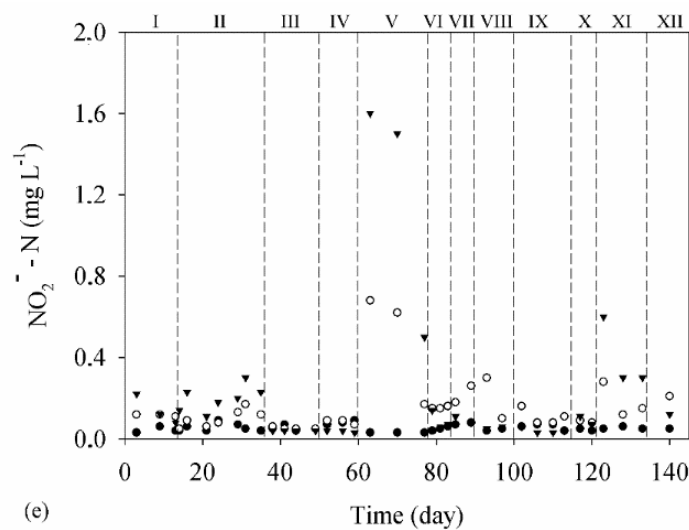
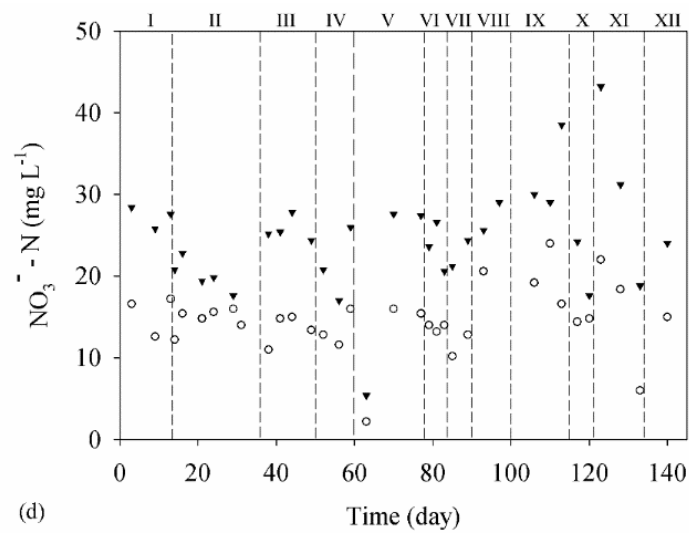
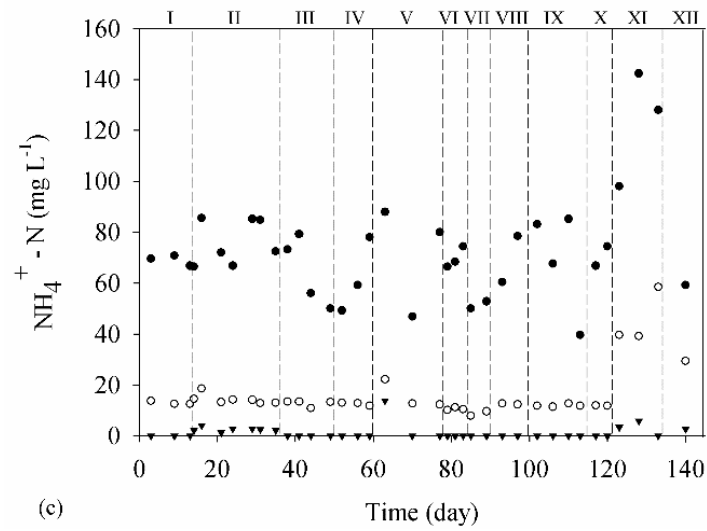


Figure 4.5 - COD (a), $\text{PO}_4^{3-}\text{-P}$ (b), $\text{NH}_4^+\text{-N}$ (c), $\text{NO}_3^-\text{-N}$ (d), and $\text{NO}_2^-\text{-N}$ (e) concentrations over the course of AGS-SBR operation. Legend: The roman numerals above the figures

indicate the phases of operation. Concentrations in reactor influent (●), after anaerobic feeding (○) and effluent (▼) are shown.

Concerning the phosphate concentration profiles, phosphorus content in the influent was maintained fairly constant throughout reactor operation (average 17 mgP L^{-1}) (Figure 4.5b). Phosphate concentrations after the anaerobic feeding period were always higher than expected based on its influent concentration and dilution in the reactor, which reveals that PAOs released phosphate. However, P-release over time was very variable after phase II. A decline in phosphate consumption during the aerated phase was noticed in phase III and became even more pronounced in phase V (after the biomass has been stored at $4 \text{ }^{\circ}\text{C}$ for 60 days, which may have contributed to this result). After this period, phosphate uptake under aeration has not been fully restored, possibly associated with an inhibitory effect caused by EDCs on PAOs. The recovery of the phosphate removal was not observed even when the EDCs were removed from the reactor influent, demonstrating the sensitivity of the enhanced biological phosphorus removal process (EBPR) to the imposed conditions. The adverse effect of pharmaceutical compounds on biological phosphate removal was reported in previous studies (AMORIM *et al.*, 2014; CASTELLANOS *et al.*, 2021). Other studies observed that emerging contaminants led to a decrease in the anaerobic phosphate release, but the effluent phosphate concentrations were unaffected (AMORIM *et al.*, 2016; KENT; TAY, 2019).

The presence of the EDCs mixture in the influent did not significantly affect ammonium removal (Figure 4.5c), implying that the AGS structure could have protected AOB from the presence of these compounds. In the first phase, ammonium was fully consumed. During phase II, a slight decrease in its removal (to around 96.7%) was observed upon EDCs supplementation. Nevertheless, it returned to 100% after interrupting the EDCs load (phase III), remaining so during phase IV. Shortly after the reactor reactivation (phase V), the effluent ammonium concentration reached more than 9 mgN L^{-1} , but AOB activity was recovered quickly, the effluent ammonium content dropped sharply and 100% removal was reached. Such performance was kept invariant throughout the next phases. The complete removal of ammonium in the subsequent shock load events reinforces the great potential of aerobic granules in keeping stable nitrification in the presence of EDCs. Moreover, even when influent ammonium concentration was doubled (120 mgN L^{-1}) during phase XI, its removal only slightly decreased, showing the

robustness of AGS system regarding nitrification. The increase in ammonium load was made to stimulate the development of nitrifying bacteria, as some authors suggest that these organisms are capable of improving the biodegradation of EDCs (JANTANAPRASARTPORN *et al.*, 2018; MARGOT *et al.*, 2016; ROH *et al.*, 2009; YI; HARPER, 2007). During this time, however, the removal of the EDCs, especially EE2, did not improve. However, it cannot be ignored that the time analysed under increasing ammonium load (phase XI) was relatively short (11 days only).

After anaerobic feeding and effluent throughout SBR operation, nitrite was observed at residual levels. Its maximum concentration in the effluent was approximately 1.6 mgN L⁻¹ at phase V (Figure 4.5e). Nitrate was the main nitrification product, being found in high concentrations in the effluent (Figure 4.5d). Similar nitrate concentrations were observed during the phases without EDCs (I, III, and V), averaging 27 mgN L⁻¹. In addition to nitrification and denitrification processes, soluble nitrogen compounds (especially ammonium) were converted into bacterial biomass via assimilation (the calculation performed to estimate nitrogen consumption for growth purposes is described in Equation 4.1). Assimilation for growth only accounted for 13% (on average) for the overall nitrogen removal. Nitrogen was mainly removed by denitrification (50.6% of TN removed by this process), except in phase VII, when assimilation (34.7%) was the main nitrogen removal route (Figure 4.6).

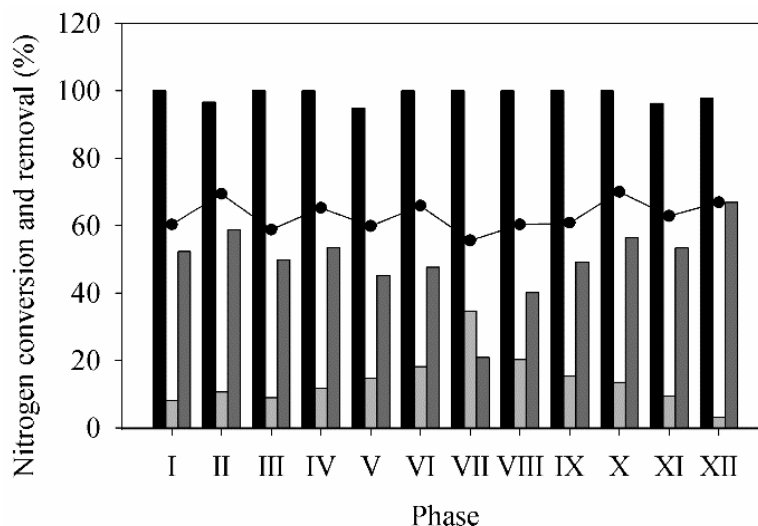


Figure 4.6 - Nitrogen removal by bacterial assimilation and denitrification. Legend: Ammonium converted into nitrite/nitrate by nitrification (black bar), nitrogen

assimilation (light grey bar), denitrification (dark grey bar) and total nitrogen removal (●).

In this study, NaCl concentration was kept constant at 12 g L^{-1} . Other researchers have previously investigated the sole effect of salt on COD, N and P removal by AGS during the treatment of saline wastewater. PAULO *et al.* (2021) showed that variation in salt levels was not detrimental to any of these processes. The authors suggested that this result was possible due to the presence of EPS-producing bacteria. In the work of LI *et al.* (2019), the performance of an AGS-SBR operating at relatively low salinity (1%) remained stable. However, at 2% and 4% salinity levels in terms of NaCl and K_2SO_4 (w/w = 1:1), the organic pollutants removal efficiencies deteriorated. P removal was impaired by salt concentrations above 21 g L^{-1} NaCl (PRONK, M. *et al.*, 2014). Other reports mentioned that the increase of salinity from 0 to 15 g L^{-1} led to significant drops in COD, N and P removal efficiency (WANG *et al.*, 2017).

4.3.3 AGS characteristics

In this study, mature AGS with a light yellow color and a smooth and compact structure, and mean diameter of 1.15 mm, was used as inoculum in the SBR. Throughout the experiment period, the granules maintained such properties. The changes in biomass characteristics evaluated through image analysis are shown in Figure 4.7. The granules were classified in IG ($0.15 \text{ mm} < D_{\text{eq}} < 1.5 \text{ mm}$) and LG ($D_{\text{eq}} > 1.5 \text{ mm}$). Diameter (Figure 4.7a) and granules average area (mm^2) (Figure 4.7b) followed the same pattern over time. The same occurred for the relative amount (%) (Figure 4.7c) of each class of granules. The total number of granules varied according to the number of IG ($0.15 \text{ mm} < D_{\text{eq}} < 1.5 \text{ mm}$), with the number of LG ($D_{\text{eq}} \geq 1.5 \text{ mm}$) remaining practically constant over the different operating phases (Figure 4.7d). The morphological parameters, such as compactness (Figure 4.7e) and robustness (Figure 4.7f), remained practically constant over time.

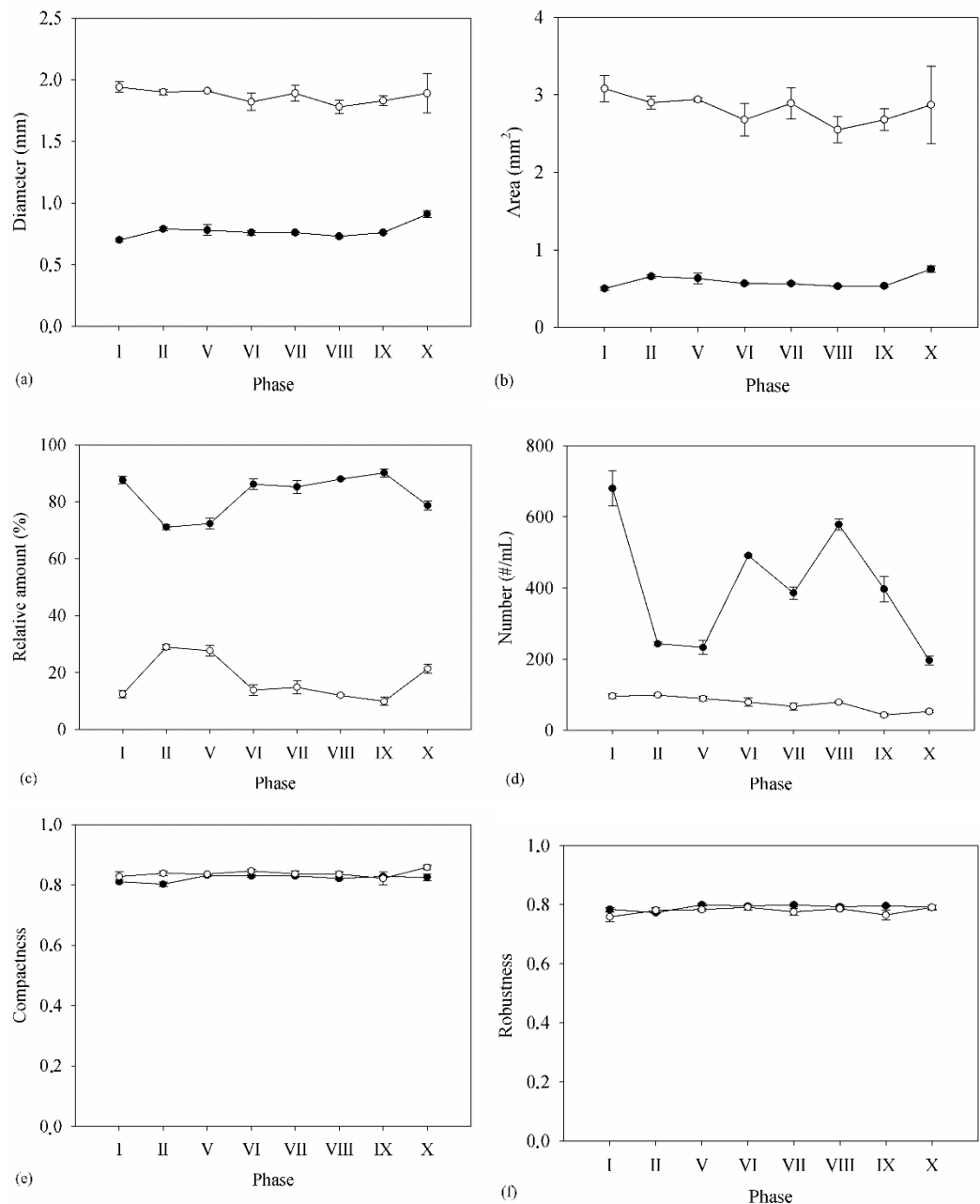


Figure 4.7 - Granules average equivalent diameter (a), area (b), relative amount (c), number of granules (d), compactness (e) and robustness (f) for large-sized (○) and intermediate-sized granules (●) throughout reactor operation.

Throughout the reactor operation, biomass was mostly composed of IG. However, a change in the size of the granular biomass from the inoculum (88% of IG) to phase II (71% of IG) occurred, remaining stable until phase V. This behaviour was not due to an increase in the amount of LG, but rather to a washout of IG, possibly due to biomass reactivation. When the EDCs feeding was restarted (phase VI), there was a slight decrease

in D_{eq} of both IG and LG (from 0.778 ± 0.045 mm to 0.756 ± 0.018 mm and 1.915 ± 0.006 mm to 1.825 ± 0.070 mm, respectively). Concomitantly, the number of IG and LG increased and decreased, respectively, resulting in a reduction in the LG relative percentage, from 28% to 14%. The decrease in the relative amount of LG continued until it reached a minimum of 10% (phase IX). Moreover, in phase X, an increase in the D_{eq} of IG (from 0.757 ± 0.006 mm for 0.914 ± 0.026 mm) and the LG relative amount to 21% was noticed. This may be associated not only with the maturation of the granules, but also with the washout of the granules with lower D_{eq} .

Figure 4.8a illustrates the solids content in the reactor over the operating time. The highest TSS values were observed during the first and last phases. In phase I, the mean TSS concentration was 15.5 g L^{-1} , with 63% corresponding to VSS, while in phase XII it amounted to 13 g L^{-1} , of which 86% was VSS (phase XII). The solids content gradually decreased until phase V, but increased after the restart period. At the beginning of bioaugmentation (phase VII), a cycle length of 12 h was adopted as an attempt to facilitate the retention of the bioaugmented bacterial strain inside the reactor. Under these conditions, an increment in the effluent TSS was observed (Figure 4.8b). The longer aerated phase may have caused the breakage of the granules due to more intense shear stress caused by aeration, leading to biomass loss from the reactor during the settling and withdrawal phase. Other authors have also reported the adverse impact of long aeration periods on the granular biomass shape and size (MOREIRA *et al.*, 2015). Sludge age was not controlled by manual sludge discharge, being therefore governed by natural solids washout (Figure 4.9). During the first four phases, the SRT varied from 25 to 15 days. After the biomass storage period (phase V), the SRT dropped to 3 days due to biomass washout, but it gradually increased until the last operating phase. The food-to-microorganism ratio ($F M^{-1}$) was lower ($0.04 \text{ gCOD gVSS}^{-1} \text{ d}^{-1}$) during the bioaugmentation period (phase VII) because of the longer aerated period (Figure 4.9).

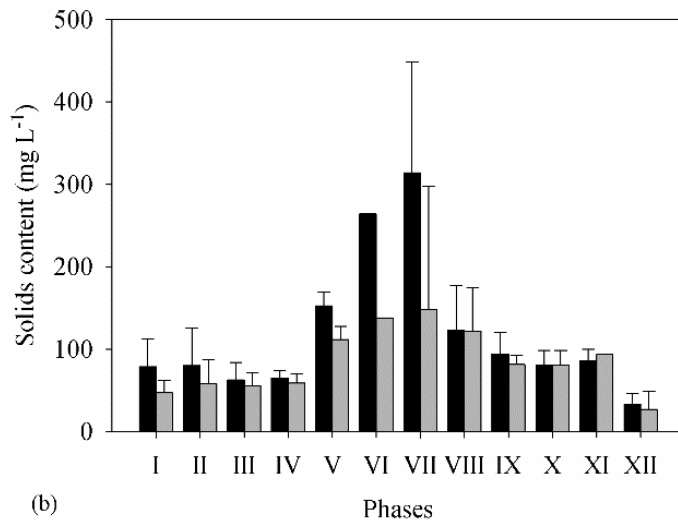
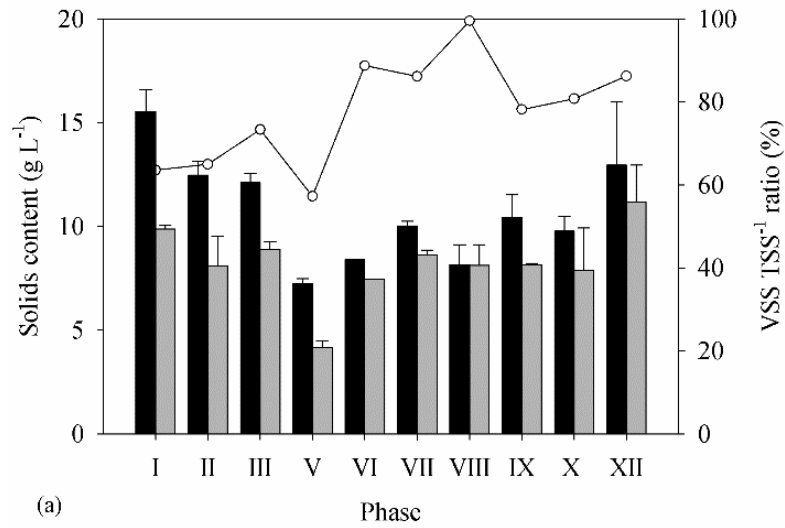


Figure 4.8 - Solids content inside the reactor (a) and in the effluent (b). VSS TSS⁻¹ ratio is also shown in the (a). Legend: TSS (black bar), VSS (grey bar) and VSS TSS⁻¹ ratio (○).

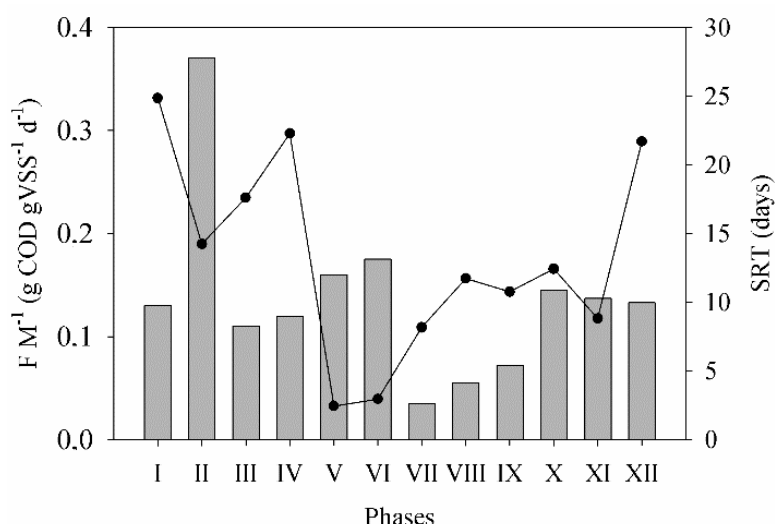


Figure 4.9 - Food-to-microorganism (grey bar) and SRT (●).

Closely related to the stability of aerobic granular sludge, EPS are usually the first barrier of microbial cells that are in direct contact with the substrates, including toxic substances, therefore interacting with them (LI *et al.*, 2015). The EPS content of the aerobic granular biomass during the operating period is shown in Figure 4.10. The concentrations of proteins (PN), polysaccharides (PS), and humic acids (HA), the main constituents of EPS, decreased from phase I to phase II and increased in phase IV. At the reactor restart (phase V), the concentrations of main EPS constituents decreased significantly when compared to the previous phase. In the following phases, their concentrations increased gradually. In phase VIII, a different profile was observed, with the PN content being greater than that of PS. In phase IX, the previous pattern was reestablished, but with a decrease in the overall EPS content. PN PS⁻¹ ratio decreased gradually from phase I (1.94) to phase VI (0.85) and increased again in phase VII (1.06) until phase IX (1.27). Previous studies have been reported that the production of EPS was an important self-protection strategy of microorganisms against toxic substances. The latter may stimulate the production of exopolymers. LI *et al.* (2015) reported that the toxicity of BPA (40 mg L⁻¹) played an important role in the production of PN. On the other hand, CYDZIK-KWIATKOWSKA *et al.* (2017) found that EPS corresponded to about 20% of granular biomass in the control reactor (no BPA) and only about 4-12% in the BPA-exposed granules (2 to 12 mg L⁻¹ BPA). Studies pointed out that high salinity tends to stimulate EPS secretion and, as a consequence, maintain good granular stability. CORSINO *et al.* (2016) have observed that EPS content increased under saline conditions compared to the control conditions without salt effect, while HOU *et al.* (2019) noticed

wide variations between PN and PS content under different salinity conditions in an AGS process. In another study, analysis of EPS components suggested the enhanced adsorption of metals by AGS might be ascribed to increasing polysaccharides content in the EPS after saline treatment (30 g L^{-1}) WU *et al.* (2019), suggesting that using salinity to increase EPS yield would be beneficial for adsorption. SHENG *et al.* (2010) also pointed out that several organic pollutants can be adsorbed on the sludge because some hydrophobic regions in EPS are responsible for increasing the biomass adsorption capacity.

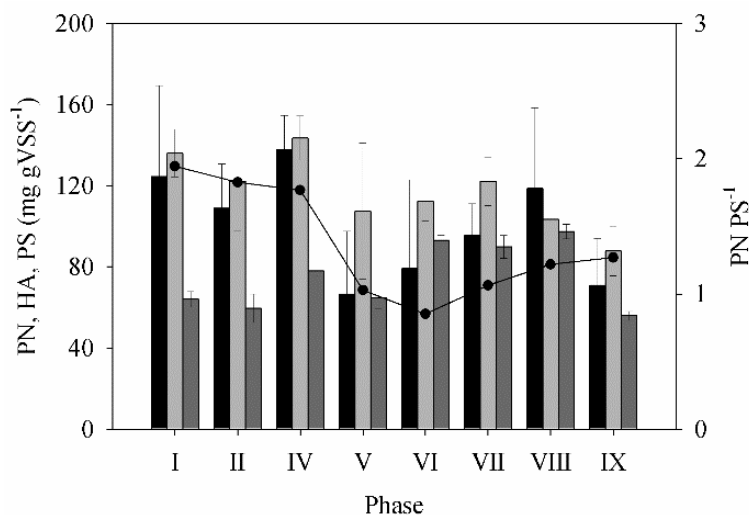


Figure 4.10 - Proteins (black bar), humic acids (medium grey bar), polysaccharides (dark grey bar), and PN PS^{-1} (●).

4.3.4 Identification of EDCs degrading strains

Using culture-dependent methods, twelve bacterial isolates were obtained from crushed granules, among which *Rhodococcus* ED55. According to BLAST results, seven strains were affiliated to Proteobacteria, one with Firmicutes and one with Actinobacteria phyla (Table 4.3). This result indicates that Proteobacteria played an important role in the degradation of EDCs. All of the bacterial isolates shared similarities between 97% and 100% with the sequences deposited in GenBank, and were closely related to isolates from environmental samples. Previous studies suggest that are certain bacterial genera, e.g., *Rhodococcus*, *Pseudomonas*, or *Sphingopyxis*, that encompasses high percentages of xenobiotic-degrading strains (STOLZ, 2009). *Labrenzia aggregata* was isolated from seawater of the East China Sea, and *Brevibacillus halotolerans* was found to have a

complex regulatory network that may help these bacteria adapt to complex environments under stressful conditions (XU *et al.*, 2019).

Table 4.3 - Phylogenetic affiliation of bacterial isolates extracted from the aerobic granules.

Isolate	Closest relative	Phylogenetic affiliation	Similarity (%)	Accession n°	Compound biodegradation (%)
E2 1^a	<i>Rhodococcus ruber</i> strain DSM 43338	Actinobacteria	99.7	NR_026185.1	100
E2 2	<i>Brevundimonas diminuta</i> strain NBRC12697	α -proteobacteria	99.8	NR_113602.1	96
E2 3^b	<i>Sphingopyxis terrae</i> strain NBRC 15098	α -proteobacteria	99.8	NR_113727.1	80
E2 4	<i>Labrenzia aggregata</i> strain NBRC 16684	α -proteobacteria	97.1	NR_113861.1	35
E2 5	<i>Pseudomonas composti</i> strain C2	γ -proteobacteria	99.8	NR_116992.1	No
EE2 1	<i>Acinetobacter johnsonii</i> strain ATCC 17909	γ -proteobacteria	99.8	NR_117624.1	No
EE2 2	<i>Pseudomonas plecoglossicida</i> strain FPC951	γ -proteobacteria	99.7	NR_024662.1	No
EE2 3^b	<i>Sphingopyxis terrae</i> strain NBRC 15098	α -proteobacteria	99.8	NR_113727.1	No
EE2 4	<i>Brevibacillus halotolerans</i> strain LAM0312	Bacilli	96.9	NR_156834.1	No
BPA 1	<i>Rhodococcus ruber</i> strain DSM 43338	Actinobacteria	99.9	NR_026185.1	22.7
BPA 2	<i>Shewanella seohaensis</i> strain S7-3	γ -proteobacteria	99.8	NR_108852.1	21.8
BPA 3	<i>Paracoccus huijuniae</i> strain FLN-7	α -proteobacteria	97.8	NR_108224.1	No

^{a, b} The strains with the same letters are equals.

From the isolated bacterial strains, four of them were able to degrade E2 (Figure 4.11a). Probably these organisms contributed to the degradation of E2 observed once this compound was added to the reactor influent wastewater. For all tests showing E2 degradation, E1 was generated and accumulated in the liquid medium (Figure 4.11b). However, the isolates were not capable of degrading E1 during the time course of the experiment. YU *et al.* (2007) indicated that only three strains of 14 isolates could degrade E1 within 7 days, after converting E2. MULLER *et al.* (2010) tested four isolates individually for estrogen removal, and only *Brevundimonas diminuta* could convert E2 to E1. For BPA, removals reached values of 22.7% and 21.8% with isolate *Rhodococcus ruber* and *Shewanella seohaensis*, respectively. While for EE2 there was no degradation. Considering the results from culture-dependent and -independent methods, it is likely the EDCs degradation by the AGS resulted from a syntrophic relationship between several isolates and non-isolated bacteria.

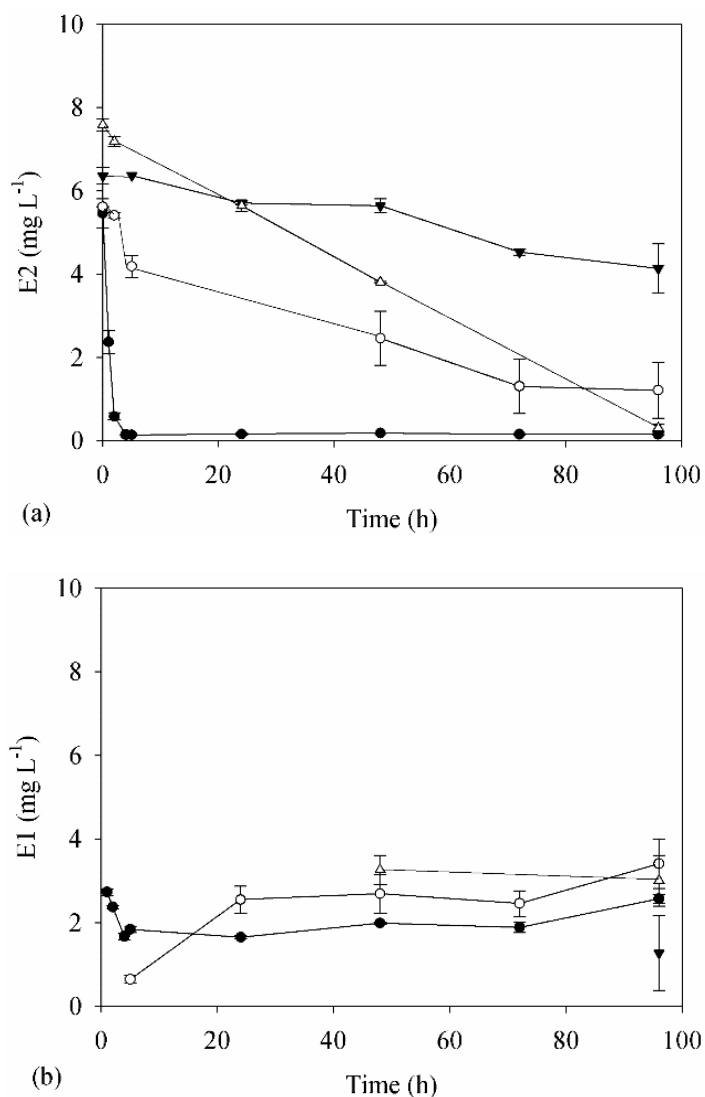


Figure 4.11 - E2 degradation by strains isolated from AGS (a) and E1 production (b). Legend: *Rhodococcus* sp. (E2 1) (●), *Brevundimonas* sp. (E2 2) (Δ), *Spingopyxis* sp. (E2 3) (○), *Labrenzia* sp. (E2 4) (▼).

4.3.5 Microbial community of the aerobic granules

4.3.5.1 DGGE analysis

DGGE analysis of the 16S rRNA gene was performed to assess bacterial community changes in aerobic granules over the reactor operating phases. The DGGE banding profiles indicated a wide bacterial diversity within the granules (Figure 4.12a). Most of the bands were common in almost all samples, while others were only present in some sampling days. A band that apparently corresponds to *Rhodococcus* sp. can be seen

on all lanes, suggesting that this bacterium was present in the biomass community since the reactor started operating, even before the bioaugmentation process. Unfortunately, it was not possible to excise the band and sequence it to confirm the identity because it was too thin.

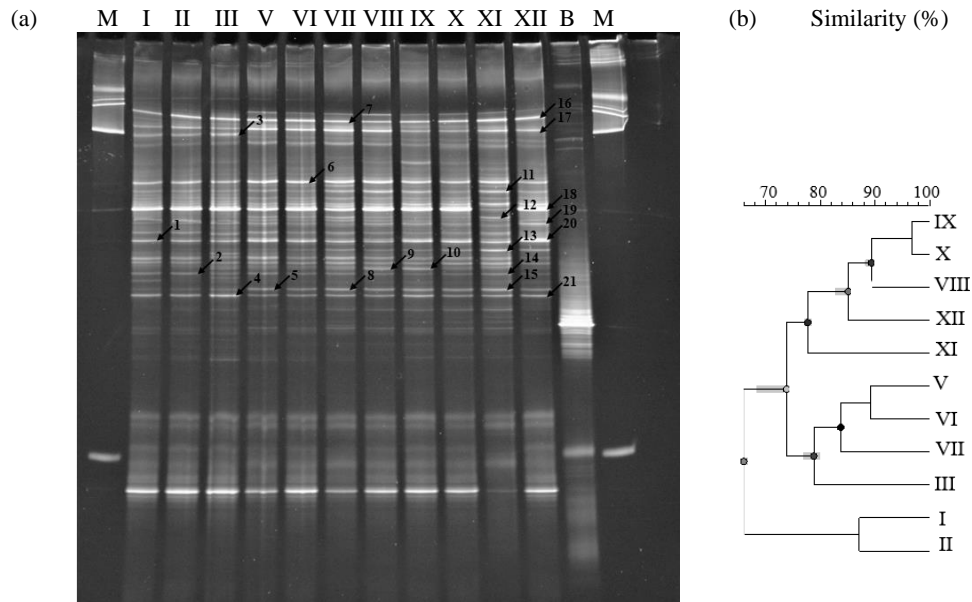


Figure 4.12 - DGGE analysis of 16S rRNA fragments of total bacterial community within the aerobic granules. (a) DGGE fingerprint of the SBR bacterial community. Different gel lanes correspond to samples collected during SBR operation (phases are indicated on the top of the lanes). Lane M: DNA marker; Lane B: EDCs degrading bacterial strain. (b) UPGMA cluster analysis of bacterial communities based on DGGE profile. Dendrogram presents the similarity, in percentage, between the DGGE samples. Similarities were calculated using the Jaccard measure.

Cluster analysis was performed aiming at characterizing the similarity between the DGGE profiles of each phase (Figure 4.12b). The dendrogram showed that samples were separated into three main clusters. One of the main clusters was composed of the initial phases of operation of the reactor (I and II), the second one comprised phases III to VII (before bioaugmentation), and the third cluster was composed of phases VIII to XII. The first cluster, which includes phase I (no EDCs fed to the reactor), presented the lowest similarity between the other clusters (around 65%). This was probably associated with the EDCs, which induced changes in the granular biomass community in the subsequent phases.

The use of indices gives more information about the microbial community. Therefore, Shannon diversity index (H), used to evaluate the diversity of bacterial communities (SHANNON; WEAVER, 1963), and the Equitability index (E), which can range from 0 (no evenness) to 1 (complete evenness) (PIELOU, 1975) was calculated. H ranged from 1.27 (phase V) to 1.54 (phase XII) (Table 4.4). Nevertheless, no significant differences ($p>0.05$) were found in the community diversity. Concerning E, similar behaviour to the diversity index (0.82 to 1.06) was observed, with no significant differences over the reactor operating period ($p>0.05$).

Seventeen different DGGE bands were excised for DNA sequencing (Figure 4.12a), from which partial 16S rRNA gene sequences were successfully retrieved and compared with sequences from GenBank (Table 4.5). Most of the bacterial 16S rDNA sequences were classified as members of Proteobacteria. Band 18, whose intensity was very strong compared to others, was present in all phases throughout reactor operation. The sequence corresponding to this band was closely associated with *Candidatus Accumulibacter*, commonly reported to be involved in P-removal.

Table 4.4 - Shannon diversity (H) and Equitability (E) indexes

Phase	H	E
I	1.38	0.96
II	1.41	0.96
III	1.30	0.84
V	1.27	0.82
VI	1.29	0.83
VII	1.34	0.88
VIII	1.37	0.91
IX	1.42	0.95
X	1.43	0.97
XI	1.34	0.87
XII	1.54	1.06

1 Table 4.5 - Phylogenetic affiliation of DGGE band DNA sequences.

Band n°.	Phylogenetic affiliation	Closest relative (accession n°.)	Similarity (%)	Isolation source
1 and 2	γ -proteobacteria	<i>Lysobacter</i> sp. strain CHu50b-3-2 (MK696268.1)	96	Sediments
3	β -proteobacteria	Uncultured Nitrosomonadales bacterium AOBseq_3b (HQ455817.1)	88	Water from ornamental fish transporting system
4 and 21	Unknown	Uncultured bacterium clone MA00070D11 (FJ772390.1)	94	Huron River /host zebra mussels
5	Chloroflexi	Uncultured Anaerolineae (AB752317.1)	88	In situ colonization system deployed in a Japanese shallow hydrothermal vent
6	γ -proteobacteria	<i>Luteimonas</i> sp. strain BO171 (MK201763.1)	89	-
7	Unknown	Uncultured bacterium isolate DGGE gel bandBUJIT2 (GQ245699.1)	89	Landfill leachate
8	Chloroflexi	Uncultured Chloroflexi bacterium clone IAFpp7125 (GU214133.1)	87	Slime sample from a papermaking mill taken at the wet section around a papermaking machine
9	Unkown	Uncultured bacterium LN875367.1)	91	Primary sludge from a tannery wastewater treatment plant
10	β -proteobacteria	<i>Janthinobacterium lividum</i> strain K5 (KP775922.1)	94	Vaginal swab /host Homo sapiens
11	Unknown	Uncultured bacterium clone OTU_61 (MN194806.1)	96	Barley (<i>Hordeum vulgare</i>) rhizosphere
12, 18 and 19	β -proteobacteria	Uncultured <i>Candidatus Accumulibacter</i> sp. Clone 38 (JQ726367.1)	98	Sequencing batch reactor for P removal
13	Bacteroidetes	Uncultured Flavobacteriaceae (AB543529.1)	83	Sewage treatment plant
14	α -proteobacteria	<i>Paracoccus</i> sp. strain JL3606 (KX989148.1)	94	Shallow-sea hydrothermal system in Kueishantao Islet
15	Chloroflexi	Uncultured Chloroflexi bacterium clone Pink_2B07 (GQ483921.1)	95	Intertidal thrombolites

Band n°.	Phylogenetic affiliation	Closest relative (accession n°.)	Similarity (%)	Isolation source
16	Unknown	Uncultured bacterium clone OTU_6613 (KX974029.1)	96	Tropical urban freshwater
17	Bacteroidetes	Uncultured <i>Cloacibacterium</i> sp. CSVI076 (MG552002.1)	81	Biofilm
20	β -proteobacteria	<i>Azoarcus</i> sp. strain HKLI-1 (MN493120.1)	97	Sewage

2

4.3.5.2 Overall bacterial community within the AGS

From the 103 assigned OTUs, 82 were shared between samples from different experimental phases, accounting for around 90.8% of all sequences. From those OTUs, 44 OTUs were detected in all samples, representing about 42.7% of the total sequences. The core microbiome of the AGS was affiliated to Proteobacteria (27 OTUs, 61.8% of all sequences), Bacteroidetes (10 OTUs, 22.7% of all sequences), Actinobacteria (4 OTUs, 4.1% of the total sequences), Acidobacteria (1 OTU, 1.0% of all sequences) and Ignavibacteriae (1 OTU, 1.0% of all sequences) phyla.

The sequences results revealed that Proteobacteria were overabundant in all phases analysed, followed by Bacteroidetes (Figure 4.13). Other phyla, such as Firmicutes, Gemmatimonadetes and Nitrospirae, showed a relative abundance lower than 0.5%. None of these were detected in all samples. Regarding class level, Betaproteobacteria was the most abundant (44.4%) in phase I (sample collected at day 1), whereas Gammaproteobacteria and Alphaproteobacteria become the predominant classes on day 77 within phase V (frequency of 23.3% and 21.5%, respectively) and on day 92, seven days after the bioaugmentation (phase VII) (frequency of 25% and 25.9%, respectively). Other class whose relative importance increased in these phases was Flavobacteriia, accounting for 17.2% (phase V) and 17.4% (Phase VII) of the total sequences, respectively. The bacterial distribution pattern in phase X was similar to that of phase I.

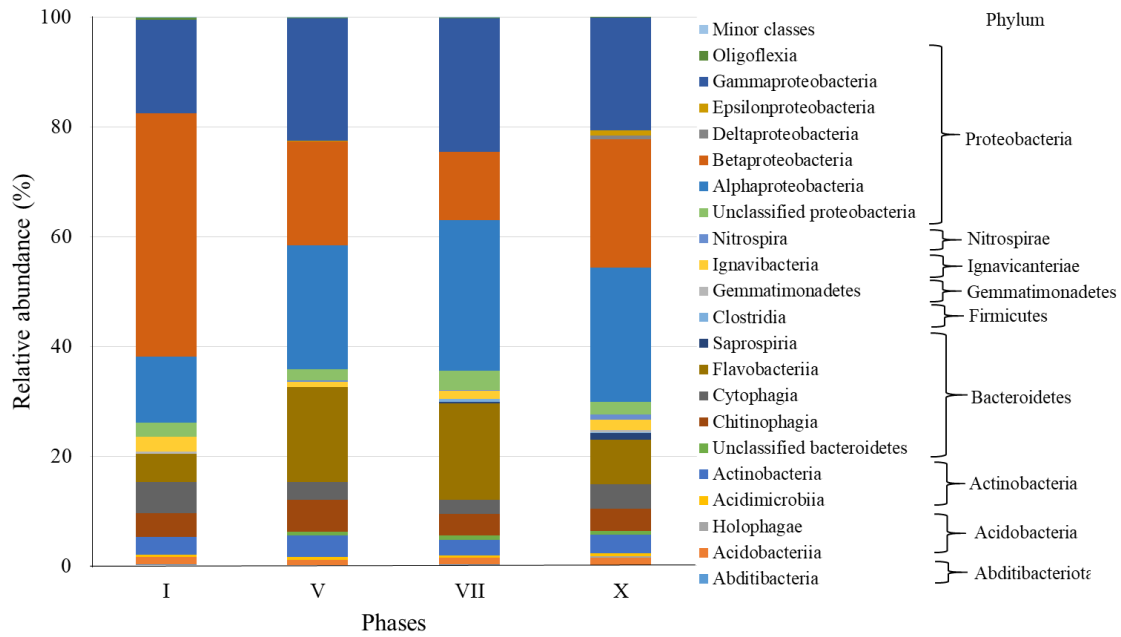


Figure 4.13 - Relative abundance of bacterial groups in class level with their respective phyla.

Rhodocyclaceae and *Xanthomonadaceae* were the most frequently detected families in the reactor, followed by *Flavobacteriaceae*, *Cytophagaceae*, *Chitinophagaceae* and *Caulobacteraceae*. Other families, e.g., *Actinobacteridae*, only appeared in lower abundance (Table 4.6).

As observed in previous studies addressing the microbial community of aerobic granules, members within Betaproteobacteria are predominant, including those closely associated with the families *Xanthomonadaceae*, *Comamonadaceae*, *Rhodocyclaceae* and *Nitrosomonadaceae*, all related to nitrification and denitrification processes (ADAV *et al.*, 2010). From them, just *Nitrosomonadaceae* was not related to EPS production (SZABÓ *et al.*, 2017).

Table 4.6 - Heatmap presenting the evolution in dominant bacterial families in the AGS biomass. Only the more abundant bacterial families were considered in this analysis.

Family	Phases				
	I	V	VII	X	
<i>Rhodocyclaceae</i>	37.69	10.05	3.98	15.52	0 to 1
<i>Xanthomonadaceae</i>	14.26	17.59	18.96	13.43	1 to 2.5
<i>Flavobacteriaceae</i>	4.65	17.15	17.55	7.63	2.5 to 5
<i>Caulobacteraceae</i>	2.79	6.72	8.21	4.22	5 to 10
<i>Chitinophagaceae</i>	4.32	5.90	3.99	4.09	10 to 20
<i>Rhodobacteraceae</i>	2.77	5.78	5.30	4.28	20 to 100
<i>Zoogloeaceae</i>	0.81	4.90	2.80	3.79	
<i>Fulvivirgaceae</i>	4.88	2.35	1.60	3.44	
<i>Phyllobacteriaceae</i>	1.65	3.16	3.34	4.22	
<i>Sphingomonadaceae</i>	1.13	3.41	4.71	2.32	
<i>Rhodanobacteraceae</i>	2.41	2.55	2.48	3.19	
<i>Microbacteriaceae</i>	2.26	2.73	2.40	2.53	
<i>Nitrosomonadaceae</i>	3.77	2.22	1.55	1.64	
<i>Comamonadaceae</i>	2.06	1.11	3.70	2.07	
<i>Chromatiaceae</i>	0.22	2.06	2.69	3.49	
<i>Rhodospirillaceae</i>	2.97	1.86	1.16	2.15	
<i>Ignavibacteriaceae</i>	2.9	0.83	1.23	1.84	
<i>Erythrobacteraceae</i>	0.00	0.00	2.15	2.79	
<i>Hyphomicrobiaceae</i>	0.00	0.55	1.39	2.82	
<i>Solibacteraceae</i>	1.44	0.88	1.17	1.29	

The heatmap with the evolution of the dominant bacterial genera in the AGS biomass (Table 4.7) shows that the frequency relative to some genera tended to increase or decrease with the addition of EDCs. Some strains were enriched in the reactor, such as those belonging to the genera *Chryseobacterium* and *Flavobacterium* within the Flavobacteriia class. For example, *Chryseobacterium*-like organisms increased from 3% (phase I) to around 12% (phase V and VII), becoming a dominant genus in AGS. However, over time, their abundance dropped to values initially found at the beginning

of reactor operation (phase I). A similar trend happened with *Flavobacterium*, but with a lower relative abundance when compared to *Chryseobacterium*. Organisms associated with *Chryseobacterium* genus were found to play a key role in tetracycline removal (WANG *et al.*, 2018), while *Flavobacterium* was dominant in AGS cultivated with pharmaceutical wastewater (AMORIM *et al.*, 2018a). *Hyphomicrobium* and *Subsaxibacter*, although not detected in phase I, showed an increasing trend over time, with frequencies reaching 2.8% and 2.6%, respectively, in phase X. *Hyphomicrobium* was reported to contribute to a significant fraction (16%) of the bacterial community in an AGS system treating pharmaceutical compounds (MUÑOZ-PALAZON *et al.*, 2021). An increasing relative abundance was also observed for microorganisms belonging to the genera *Mesorhizobium* and *Thiolamproyum*, likely due to their capability to adapt to the presence of EDCs.

The relative abundance of *Chryseolinea* genus decreased from 4.4% in phase I to 2.1% and 1.4% in phases V and VII, respectively, but increased to 2.9% in phase X. This behaviour may indicate that organisms within this genus were adversely affected by the EDCs mixture. However, the increase in the relative abundance suggests the adaptation of these organisms. *Rhodococcus* sp. was detected on day 92, eight days after bioaugmentation of the AGS system, and represented 0.4% of the total bacterial community. However, on day 120 (phase X), it was no longer observed in the bioreactor.

The sum of the relative abundance of the main genera associated with important functional groups within the AGS system is shown in Table 4.8. *Rhodocyclus* genus, closely associated with one of the main PAOs, predominated over other genera in phase I (37%). However, its relative frequency substantially decreased in subsequent phases to 9% (phase V) and 3% (phase VII), with a slight increase being observed in phase X (14%). This result was accompanied by a decline in phosphate consumption during the aerated phase from phase III, which became more pronounced in phase V. After this period, phosphate uptake under aeration has not been fully restored. Although at low relative abundance, other bacteria associated to P-removal were observed during the reactor operation. *Tetrasphaera*-like organisms were detected in phases I, V and X, but not in phase VII. *Gemmatimonas*, also reported to be involved in P-removal (PAULO *et al.*, 2021), were present just in phases I and X, with a relative abundance of 0.5% and 0.4%, respectively.

Table 4.7 - Heatmap presenting the evolution in dominant genera in the AGS biomass. Only the more abundance bacterial genera were considered in this analysis.

Genus	Phase				
	I	V	VII	X	
<i>Rhodocyclus</i>	37.7	9.5	3.1	14.2	0 to 1
<i>Lysobacter</i>	9.8	7.9	8.7	7.1	1 to 2.5
<i>Chryseobacterium</i>	2.9	12.3	12.1	3.3	2.5 to 5
<i>Brevundimonas</i>	2.8	6.7	8.2	4.2	5 to 10
<i>Edaphocola</i>	4.3	5.9	3.7	3.9	10 to 20
<i>Azoarcus</i>	0.8	4.9	2.8	3.6	20 to 100
<i>Chryseolinea</i>	4.4	2.1	1.4	2.9	
<i>Frigoribacterium</i>	2.3	2.7	2.4	2.5	
<i>Mesorhizobium</i>	0.9	2.3	2.5	3.5	
<i>Paracoccus</i>	0.6	3.4	3.4	1.9	
<i>Nitrosomonas</i>	3.8	2.2	1.5	1.6	
<i>Thiolamprovum</i>	0.2	2.1	2.7	3.5	
<i>Pseudofulvimonas</i>	2.0	2.1	2.1	2.7	
<i>Hydrogenophaga</i>	1.8	0.8	3.3	1.7	
<i>Flavobacterium</i>	0.9	2.7	2.6	0.9	
<i>Defluviicoccus</i>	2.3	1.8	1.0	1.8	
<i>Ignavibacterium</i>	2.7	0.8	1.2	1.8	
<i>Candidatus Solibacter</i>	1.4	0.9	1.2	1.3	
<i>Mariniflexile</i>	0.8	1.6	1.6	0.8	
<i>Hyphomicrobium</i>	0.0	0.5	1.3	2.8	
<i>Subsaxibacter</i>	0.0	0.5	1.3	2.6	
<i>Rhodobacter</i>	0.7	1.3	0.9	1.1	
<i>Erythrobacter</i>	0.0	0.0	1.2	1.6	
<i>Denitromonas</i>	0.0	0.5	0.9	1.3	
<i>Tetrasphaera</i>	0.5	1.1	0.0	0.9	
<i>Nitrobacter</i>	0.3	0.5	0.4	0.9	
<i>Nitrospira</i>	0.0	0.3	0.2	1.1	

Nitrifying bacteria were identified in all biomass samples (Table 4.8). The relative frequency of *Nitrosomonas*, a well-known AOB, gradually decreased from 3.8% (phase I) to 2.2% (phase V), 1.5% (phase VII) and 1.6% (phase X). Nevertheless, ammonium removal capacity was not adversely affected when the AGS reactor was exposed to the EDCs load. The relative abundance of nitrite oxidizers of the genus *Nitrobacter* increased over time, from 0.3% (phase I) to 0.9% (phase X). It has been reported to be the dominant NOB in biological wastewater treatment systems (VÁZQUEZ-PADÍN *et al.*, 2009). *Nitrospira*, another nitrite oxidizer, was not identified only in phase I. NOB was present in lower relative abundance when compared to AOB. Despite their significant contribution to nitrogen turnover, the low relative abundance and diversity of nitrifying bacteria were already reported in previous studies (SZABÓ *et al.*, 2017). The adverse impact of pharmaceuticals on nitrifying activity has also been reported, being the adverse effect more intense on NOB (AMORIM *et al.*, 2018).

Denitrifying bacteria had the higher relative abundance among the bacterial functional groups in all phases analyzed, except during phase I (Table 4.8), indicating that this group was dominant and possibly relevant for the EDCs removal. *Denitromonas*, reported as dominant heterotrophic denitrifiers in aerobic granular biomass with a major role in denitrification under saline conditions (YAO *et al.*, 2021), was also detected from phase V (0.5%) onwards. Its relative abundance increased over the experiment (0.8% and 1.4% for phases VII and X, respectively).

Table 4.8 - Relative abundance (%) of the main genera associated with important functional groups within the AGS system. The sum of each category is shown in bold.

Functional group	Genus	Phases			
		I	V	VII	X
PAO	<i>Rhodocyclus</i>	37.7	9.5	3.1	14.2
	<i>Tetrasphaera</i>	0.5	1.1	0.0	0.9
		38.2	10.6	3.1	15.1
GAO	<i>Defluviicoccus</i>	2.3	1.8	1.0	1.8
Nitrifying bacteria	<i>Nitrosomonas</i>	3.8	2.2	1.5	1.6
	<i>Nitrobacter</i>	0.3	0.5	0.4	0.9
	<i>Nitrospira</i>	0.0	0.3	0.2	1.1
		6.4	4.8	2.1	3.6
Denitrifying bacteria	<i>Lysobacter</i>	9.8	7.9	8.7	7.1
	<i>Hydrogenophaga</i>	1.8	0.8	3.3	1.7
	<i>Azoarcus</i>	0.8	4.9	2.8	3.6
	<i>Mesorhizobium</i>	0.9	2.3	2.5	3.5
	<i>Paracoccus</i>	0.6	3.4	3.4	1.9
	<i>Flavobacterium</i>	0.9	2.7	2.6	0.9
	<i>Rhodobacter</i>	0.7	1.3	0.9	1.1
	<i>Denitromonas</i>	0.0	0.5	0.9	1.3
	14.9	23.8	25.1	21.1	

4.4 Conclusion

In this study, the effect of EDCs shock loading events on AGS-SBR performance and microbial community structure were assessed. E2 was efficiently biodegraded by the system, with 60% to 80% being converted anaerobically, and the remaining quickly consumed in the aerated phase. BPA was biodegraded after bioaugmentation with EDCs-degrading bacterial strain, while adsorption/desorption of EE2 onto aerobic granular biomass was found. EDCs shock loads did not significantly affect COD and ammonium removal, whereas phosphate removal was adversely affected. Nevertheless, AGS structure may have protected the microbial community from the toxic effects of EDCs, leading to the maintenance of the carbon, nitrogen and phosphorus conversions.

Molecular analyses showed that the presence of EDCs mixture led to dynamic changes in the microbial community profile, with increases in the relative abundance of *Chryseobacterium* and *Flavobacterium*. PAOs were severely affected by EDCs, corroborating the P removal performance results. Nevertheless, the results pointed to the presence of a core microbiome, which was maintained over reactor operation.

5. CONCLUSION

This thesis allowed a better understanding of the removal mechanisms of some target emerging contaminants (E2, EE2 and BPA) in aerobic granular sludge (AGS) systems. By addressing the microbial community activity and diversity, the bacterial groups most affected by the presence of EDCs and those that most contributed to their removal were evaluated.

In the first study, the objective was to understand the main removal mechanism of the EDCs in aerobic granular biomass, revealing the contribution of adsorption and biodegradation to the removal of the target compounds in the different phases of an SBR cycle operated under alternating anaerobic-aerobic conditions. An estimate was possible through the extraction of compounds that were sorbed in the granular biomass at the end of each phase.

In the second study, a different approach was taken. Besides evaluating the saline condition ($12 \text{ g L}^{-1} \text{ NaCl}$) in wastewater on the removal of EDCs, the compounds were fed to the reactor in shock load in only one cycle per day. A specific EDCs-degrading bacterial strain was added to the system aiming at improving the removal of such compounds.

A similar pattern was observed regarding EDCs removal. E2 was completely consumed, being initially sorbed on the granular biomass during the anaerobic period of the sequencing-batch reactor (SBR). The remaining E2 in the liquid and that previously adsorbed was released and quickly biodegraded under aeration. BPA was not readily metabolized, but its removal improved over time, being fully biodegraded. In one study, BPA removal was attributed to bioaugmentation with an EDCs-degrading bacteria. However, BPA was also found to be completely removed without the bioaugmentation. Despite being initially toxic to the microorganisms, long-term operation allowed acclimation to this compound and its utilization as a carbon source. EE2 was sorbed onto the granular sludge biomass in the anaerobic feeding period of the SBR cycle in a lower proportion than that of the other compounds. In the aerated period, EE2 was desorbed from the granular biomass, but the microorganisms could not consume it.

The EDCs supplementation did not significantly affect the COD removal. In the first study, the nitrification process was affected, because NOB could not convert nitrite to nitrate efficiently, which was not observed in the other study. The activity of PAOs

was affected in both works, probably caused by the presence of EE2. Moreover, significant shifts in the microbial community were noticed over the experiments. The main bacterial genera were different for each study, possibly due to the different salinity levels and HRT to which the AGS was subjected. For the saline condition ($12 \text{ g L}^{-1} \text{ NaCl}$), the introduction of the EDCs mixture increased the relative abundance of *Chryseobacterium* and *Flavobacterium*, while in the non-saline synthetic domestic wastewater, the frequency of *Methylophilus* and *Pseudomonas*-related organisms increased, likely playing an important role in the EDCs removal. Moreover, a change in the microbial community was observed, with a change in the predominance of the genus *Klebsiella* to *Propionivibrio* throughout the phases. Besides that, high microbial diversity was sustained over reactors operation, which may be related to the stability of the AGS process during EDCs loading.

Overall, the AGS process showed satisfactory results concerning the simultaneous removal of organic matter, nutrients, and emerging contaminants (E2 and BPA). Despite adsorption being important, especially during anaerobic conditions, desorption of compounds occurred during the sequencing batch reactors (SBR) cycles, indicating that biodegradation was the main mechanism for removing the compounds in both anaerobic and aerobic periods of the SBR cycle. More recalcitrant compounds, such as EE2, can be toxic to the microorganisms and compromise the removal of nutrients, especially phosphate. A longer acclimation time may be necessary for these cases. Furthermore, alternatively, optimizing the reactor operating conditions to favour the degradation of EE2 could be interesting. With the extension of the aerated period and, consequently, the increase in the wastewater retention time inside the reactor, the strategy would provide more time for the microbial community to assimilate the compound.

Research topics for future studies are suggested below:

- Evaluate the removal of other emerging contaminants found in domestic wastewater and aquatic environments by AGS. That could bring specific substance-related perceptions about the performance and microbial community dynamics of aerobic granules;
- Assess EDCs concentration found in the environmental samples (ng L^{-1} and $\mu\text{g L}^{-1}$) to confirm the observed removal mechanisms for higher concentrations;

- Refine the methodology for extracting compounds from granular biomass since only an estimate of the adsorbed amount was possible;
- Increase the exposure time of AGS to EDCs to evaluate if the microbial community will acclimate to EE2 and, consequently, be able to metabolize it;
- Favor the development of denitrifying bacteria, since they were important for the metabolism of EDCs;
- Optimize reactor operating conditions to promote de biological degradation of more recalcitrant compounds, such as EE2.

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APÊNDICE

A. IMPACT OF THE PRESENCE OF AIR DURING THE ANAEROBIC FEEDING PERIOD OF AN AEROBIC GRANULAR SLUDGE REACTOR: IMPLICATIONS ON BIOMASS STABILITY AND TREATMENT PERFORMANCE

A.1 Introduction

Aerobic granular sludge (AGS), a self-immobilized microbial consortium containing different functional microorganisms, is a promising technology for wastewater treatment. Compared with conventional activated sludge (CAS), AGS has excellent settleability, strong shock resistance, dense and strong structure, besides allowing high biomass retention within the bioreactor (ADAV *et al.*, 2008; WEISSBRODT *et al.*, 2013). Aerobic granules represent a complex ecological system where different spatial structures and microbiological niches are formed due to substrate and oxygen gradients between the surface and interior of the granule (De KREUK *et al.*, 2006; LOCHMATTER *et al.*, 2013). Such feature enables simultaneous organic matter, nitrogen and phosphorus to be removed in a single reactor unit, usually operated as a sequencing batch reactor (SBR) to facilitate granules development and maintenance (BASSIN, 2018).

Despite these attractive characteristics, AGS process is not free of failure. One of the main technical problems encountered during the operation AGS systems is the instability of aerobic granular biomass, often caused by filamentous bacteria overgrowth. It has been stated that low levels of filamentous growth do not cause operational problems and may even stabilize the granule structure (XIA *et al.*, 2018). However, once filamentous organisms dominate the microbial community, AGS settling properties deteriorate, and subsequent biomass washout occurs (LIU; LIU, 2006). Some operating parameters have already been associated with this instability, such as substrate composition, aeration intensity, long solid retention times (SRT), high temperatures, and the dynamics of the organic substrate conversion over the SBR cycle (BASSIN *et al.*, 2019; FIGUEROA *et al.*, 2014; ZHOU *et al.*, 2014). The latter is of utmost importance for the formation of stable AGS.

Overall, AGS-SBR operation is conducted in a feast-famine regime, with the former being carried under anaerobic conditions to favour bacteria that can store the available organic substrate in the form of intracellular polymers polyhydroxyalkanoates (PHA). Polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) are the two most important representative organisms that show this behaviour in biological wastewater treatment systems (de KREUK; van LOOSDRECHT, 2004). Through this operation pattern, the growth of fast-growing heterotrophic bacteria, which often leads to sludge instability and, ultimately, degranulation (BASSIN *et al.*, 2019; de KREUK; van LOOSDRECHT, 2004), is not stimulated, as they need an external electron acceptor for energy generation (BASSIN *et al.*, 2019). Therefore, to keep the granular biomass stable in the long term and ensure a good treatment performance to comply with environmental regulations, it is essential to choose appropriate reactor operating conditions. As mentioned before and stated by many previous studies ((ADAV *et al.*, 2010; BASSIN, 2018), it is essential to maintain the feast-famine condition, with substrate present in the anaerobic phase and few substrate available in the aerated period to prevent the development of fast-growing heterotrophs and filamentous outgrowth.

In order to inhibit the growth of filamentous organisms or recover biomass after granule fragmentation, some studies have been developed. Metal ions are beneficial for accelerating the granulation process and enhancing granular strength. Ca^{2+} and Mg^{2+} divalent cations (10 mg L^{-1}) were found to positively affect the granulation process (LI *et al.*, 2009). Iron-containing compounds have also been used as chemical agents to improve sludge settleability. AGRIDIOTIS *et al.* (2007) found that 30 mg L^{-1} of Fe^{2+} helped convert filamentous structures into a compact structure, improving activated sludge settleability. YILMAZ *et al.* (2016) demonstrated that the addition of iron ions increased the size and stability of AGS. CAI *et al.* (2018) showed that the constant Fe^{2+} supply is more beneficial than the pulse dosing strategy for biomass growth, achieving granules with higher bioactivity. MOURA *et al.* (2018) combined iron addition and enhanced aeration with prolonged shear stress. Such strategy led to significant improvements in the morphology of the granules, whose stability was recovered after filamentous outgrowth.

Despite some previous studies that have reported on the instability in aerobic granular systems and how to remediate and/or prevent it, few works described how the system performance is impacted if an operational issue takes place. Operational problems are a common reality in wastewater treatment plants (WWTPs), and it is very important to know

how the system will respond to the disturbance and how it can be handle. In this work, it was investigated how an operational problem represented by the presence of air during the anaerobic feeding phase in some cycles of the AGS-SBR affects the stability of the granular biomass and the performance of the reactor concerning the simultaneous conversions of carbon, nitrogen, and phosphorus. Strategies to overcome the adverse impacts associated with such disturbance were also addressed, seeking to propose simple and easily implementable solutions to circumvent such problems.

A.2 Materials and methods

A.2.1 Experimental set-up and operating conditions

An AGS bubble column-type SBR was continuously monitored for 212 days. The reactor was made of Plexiglass in cylindrical format with 5 cm of internal diameter, 79 cm of useful height, totalizing 1.5 L of working volume. Each operating cycle of the SBR lasted 3 h (180 min) and was composed of feeding under anaerobic conditions in an upflow mode from the bottom of the reactor (60 min), aeration (112 min), biomass settling (3 min) and effluent withdrawal (5 min). Eight cycles of 3 h were operated daily, with 0.95 L of influent being fed and discarded each cycle. Therefore, a volume exchange ratio of 63% was applied, leading to a hydraulic retention time (HRT) of 4.7 h. The system was controlled by a programmable logic controller that activated/deactivated the pumps of influent feeding, effluent discharge and solenoid valves that allowed the passage of compressed air. Air was provided at the bottom of the reactor through a porous diffuser. Dissolved oxygen (DO) remained approximately constant at 6 mg/L during the aeration phase of the SBR, while pH was kept at 7.5. The bioreactor was operated at room temperature ($25 \pm 2^\circ \text{C}$). The AGS biomass used as inoculum was taken from another lab-scale reactor running in a similar set-up. So, the granulation step was not necessary, minimizing the start-up time.

In order to obtain synthetic wastewater with a typical composition found in domestic wastewater (COD $\sim 400 \text{ mg L}^{-1}$, ammonium $\sim 50 \text{ mgN L}^{-1}$ and phosphate $\sim 15 \text{ mgP L}^{-1}$) (METCALF; EDDY, 1991), 150 mL of two solutions prepared and stored separately were mixed with 650 mL of tap water before being fed to the reactor. Based on previous work (Bassin et al., 2012), one solution (Solution 1) contained the organic carbon source ($\text{NaCH}_3\text{COO}\cdot 3\text{H}_2\text{O}$ 38.1 mM, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 1.29 mM, KCl 4.8 mM and $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.41

mM), and the other (Solution 2) comprised the nutrients source (NH₄Cl 21.4 mM, K₂HPO₄ 2.1 mM, KH₂PO₄ 1.1 mM). The latter was also amended with a trace element solution (VISHNIAC; SANTER, 1957), added in a proportion of 5 mL L⁻¹.

The operation of the reactor was divided into five phases (Table A.1). During phase I, the reactor was running stably, without any interference. After that, a disturbance, referred to as DI, happened. DI was characterized by the unintentional introduction of DO by injecting air into the bottom of the reactor during anaerobic feeding in about 20 SBR cycles during four consecutive days. This was caused by a sealing problem in the solenoid valve responsible for releasing or restricting the passage of compressed air to the reactor. Then, a recovery period with air addition suppression and iron ions supplementation (II) was initiated. During this phase, FeCl₃ was added to Solution 2 to obtain a concentration of 10 mgFe³⁺ L⁻¹ as a strategy to control and eliminate filamentous bacteria (MOURA *et al.*, 2018). Subsequently, the reactor was subjected again to the introduction of DO during anaerobic as a result of the solenoid valve malfunction. In order to avoid exposing the granules to several cycles with the undesirable condition of aeration within the aerobic feeding due to the operational problem in the solenoid valve responsible for releasing and restricting the passage of compressed air to the reactor, the granular biomass was withdrawn from the reactor and stored at 4 °C for three days. This period was referred to as a second disturbance event (DII).

After the biomass storage period, normal operation (III), without the addition of Fe³⁺ was implemented. Later, the addition of Fe³⁺ to the reactor influent was implemented again (IV), but it was added to the feeding tank containing tap water instead of Solution 2, as made in phase II, to avoid chemical phosphorus precipitation and therefore reduction of influent phosphate concentration. After 35 days, Fe³⁺ was removed from the system, and a stable operation period was reestablished (V).

Table A.1 – Operating phases of the SBR before and after the disturbances period.

Phase	Days	Conditions
Stable operation (I)	0 - 88	Before the first disturbance period
Disturbance (DI)	89 – 92	Air was introduced during the anaerobic feeding period
Recovery with the addition of iron ions (II)	93 – 129	FeCl ₃ was added to Solution 2 (10 mgFe ³⁺ L ⁻¹)

Phase	Days	Conditions
Disturbance (DII)	130 - 133	Granules were withdrawn from the reactor
Normal operation (III)	134 - 143	Normal operation of the reactor (without adding Fe ³⁺)
Return of iron ions (IV)	144 - 179	Return of Fe ³⁺ addition (added to the tap water tank)
Stable operation (V)	180 – 212	Removal of Fe ³⁺

A.2.2 Analytical measurements

Samples were regularly collected at the influent, after the anaerobic feeding and effluent, and were filtered through nitrate cellulose (0.45 mm pore-size) to remove any solids. Inorganic soluble nitrogen (as ammonium, nitrite and nitrate), phosphate and COD were measured by colorimetric methods, following the protocols described in the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The methodology described by PRONK *et al.* (2014) was used to determine total (TSS) and volatile (VSS) suspended solids within the AGS-SBR. Extracellular polymeric substances (EPS) were analyzed as total protein (PN) and polysaccharides (PS), and were extracted from the granular biomass by heating method (BASSIN *et al.*, 2012). The determination of PS and PN concentrations was assessed based on the method proposed by DUBOIS *et al.* (1956) and BRADFORD (1976), respectively.

The biomass sedimentation capacity was measured by the sludge volume index (SVI) test in a graduated cylinder, according to SCHWARZENBECK *et al.* (2004), while the biomass density was determined by the pycnometer method (WINKLER *et al.*, 2012). The physical appearance of the granules was assessed by a ZEISS Stemi 508 stereoscope. The average diameter of the granules was determined by image analysis through Image J software. The theoretical settling velocity of the granular particles was calculated taking into account density and average diameter data. For Reynolds number of the particle (Re_p) < 1, Stokes's

law was used: $v_s = \frac{g}{18} \frac{\rho_p - \rho_w}{\rho_w} \frac{d_p^2}{v_w}$; in the range $1 < Re_p < 10^3$, the sedimentation velocity was

calculated by the coefficient of resistance $c_w(\text{Re}_p) = \frac{24}{\text{Re}_p} + \frac{4}{\sqrt{\text{Re}_p}} + 0.34$ and then solved the

$$\text{equation: } v_s = \sqrt{\frac{4}{3} d_p \frac{\rho_p - \rho_w}{\rho_w} \frac{g}{c_w(\text{Re}_p)}}.$$

Where v_s is sedimentation velocity of a single particle (m s^{-1}), d_p granule diameter (m), ρ_p and ρ_w granular and water density (Kg m^{-3}), respectively, g gravitational constant (9.8 m s^{-2}), ν_w kinematic viscosity water ($10^{-6} \text{ m}^2 \text{ s}^{-1}$), $c_w(\text{Re}_p)$ coefficient of resistance and Re_p Reynolds number.

On the other hand, the experimental settling velocity was measured in a graduated cylinder filled with tap water. The time (t) required for the average of granules of different sizes to travel a distance equivalent to the height of the column ($d = 0.29 \text{ m}$) was recorded and then, the settling velocity of granules was determined by the equation: $v_s = \frac{d}{t}$.

A.2.3 Assimilation of nitrogen

The amount of incorporated ammonium nitrogen (N_{ass}) (gN/d) for cell growth was estimated according to the modified equation (WAN; BESSIÈRE; SPÉRANDIO, 2009):

$$N_{\text{ass}} = f_N \times \text{VSS}_{\text{eff}} \times \text{FR} \quad (\text{A.1})$$

Where f_N is the fraction of nitrogen in the sludge ($0.12389 \text{ mgN mgVSS}^{-1}$); FR is the flow rate (L d^{-1}); VSS_{eff} is the concentration of solids in the final effluent (gVSS L^{-1}).

A.3 Results and discussion

A.3.1 AGS characteristics

The AGS-SBR operation was monitored for 212 days. From the 1st until the 88th day, the reactor operation was well controlled, allowing stable granules and high organic matter and nutrient removal efficiency. During the stable operation, the granular biomass established in the reactor had a light color, with the predominance of round granules showing no apparent filaments (Figure A.1a) and a diameter between 1 and 2 mm (Figure A.2).

From day 89 to 92, however, air was unintentionally introduced to the reactor in several cycles during the feeding period (which is normally carried out under anaerobic conditions) due to an operational problem with the solenoid valve. As a result, an outgrowth of filamentous bacteria was observed, compromising the system stability and deteriorating effluent quality. Two days after air introduction during the anaerobic feeding, filamentous organisms started to appear (Figure A.1b). In order to control the growth of filamentous microorganisms, iron was added to the influent at a concentration of $10 \text{ mg Fe}^{3+} \text{ L}^{-1}$ (Phase II), as recommended by previous studies (MOURA *et al.*, 2018). However, even with the iron supply for five days, filamentous granules remained in the reactor. Nevertheless, the formation of new granules was noticed. Because of the predominance of filamentous bacteria, an apparent increase in particle diameter was observed (Figure A.1c).

After 30 days of iron ions addition, the filaments were detected in smaller amounts, concomitant with the formation of new granules (Figure A.1d), confirming the gradual recovery of the biomass. However, on day 130, a malfunction of the solenoid valve allowed air to enter the SBR again during the anaerobic feeding period. To avoid another proliferation of filaments event from occurring due to biomass exposure to DO in the feast phase (high COD availability), the granules were removed from the reactor and stored for three days at 4°C (DII). Although this period was relatively short, Figure A.1e shows that more filaments reappear in the granular biomass. Thus, iron was added again for 35 days to the influent wastewater (phase IV) in order to overcome this situation. In phase V, compact granules without filaments and with a diameter between 1 and 2 mm were obtained (Figure A.1f).



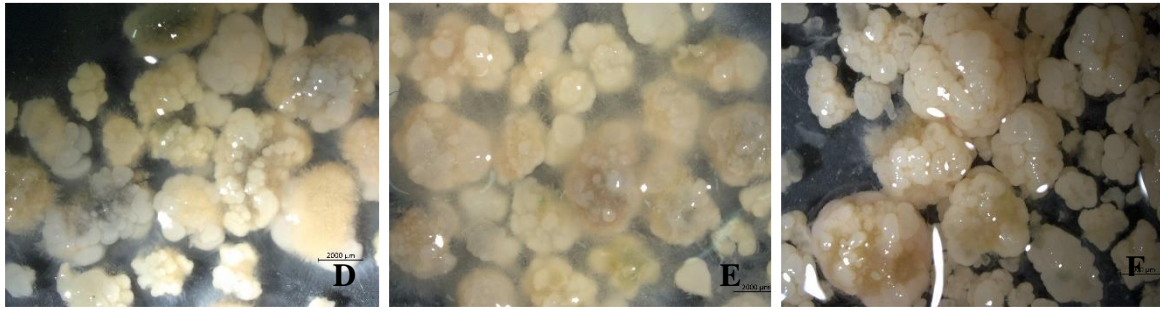


Figure A.1 – Images of granules during the experimental phases: stable conditions (phase I) (a), disturbance (DI) (b), five days (c) and thirty days (d) after Fe^{3+} supply (phase II), phase IV (e) and phase V (f).

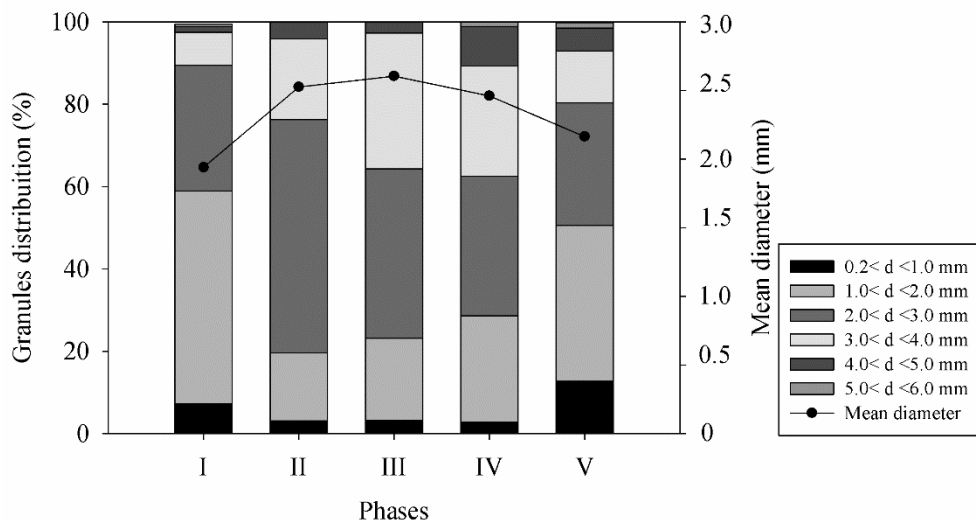


Figure A.2 - Granule size distribution expressed as a percentage relative to the total biomass in the reactor and average granule diameter.

During the first stable operation period (phase I), the sludge age was controlled at around 15 days by manual disposal of excess sludge. Figure A.3a shows the reactor solids content and the solids retention time (SRT) for each phase of SBR operation. During phase I, the average TSS concentration within the SBR was 8.0 g L^{-1} , of which 69.9% corresponded to VSS. Despite occurring for only 4 days, the introduction of air during the anaerobic feeding phase (period DI) already caused the appearance of filamentous bacteria, which altered the physical characteristics of the granules. In fact, after this disturbance, soft and fluffly granules became predominant, and a considerable fraction of the granular biomass ended up being carried along with the treated effluent (Figure A.3a and b). As a result, a substantial reduction in the reactor solids content was noticed, with TSS, VSS and FSS concentrations assuming

values around 3.24, 2.70 and 0.55 g L⁻¹, respectively (Phase III). Concomitantly, the sludge age decreased from 21 to 6 days without any intentional sludge withdrawal (Figure A.3b).

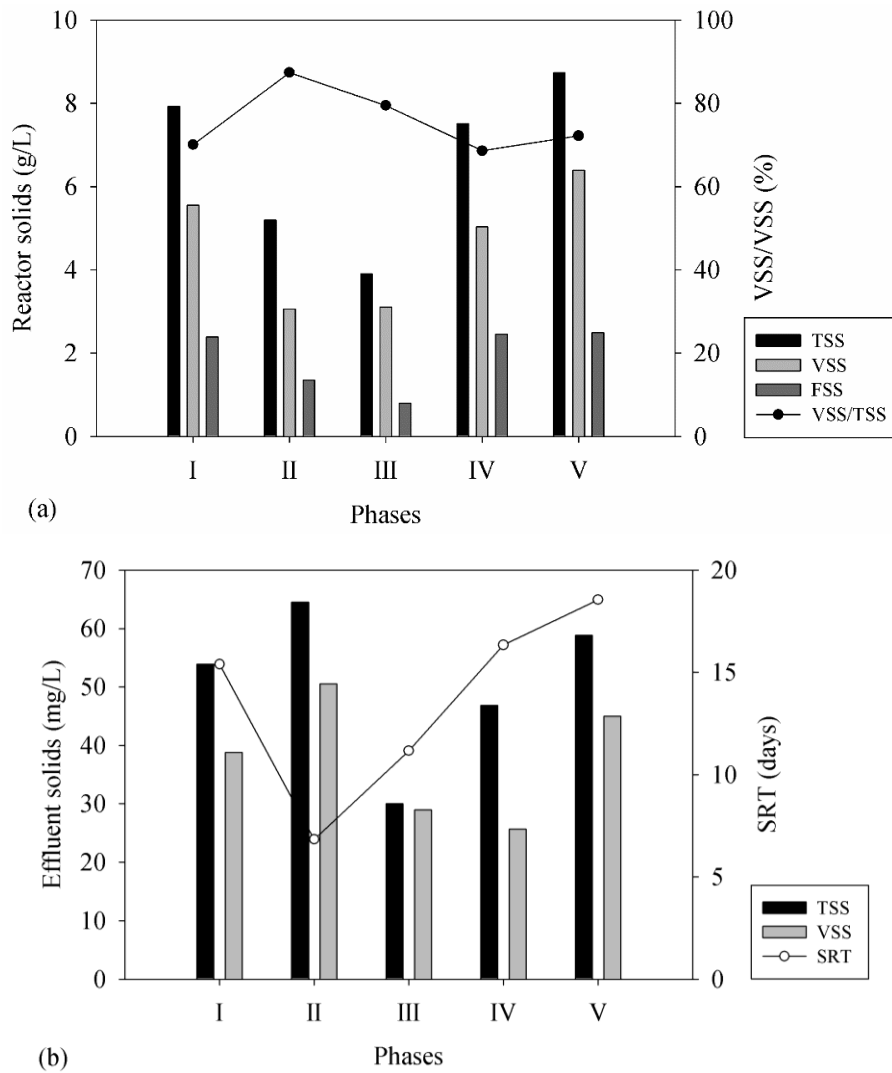


Figure A.3 - Reactor solids (a) and effluent solids and the SRT (b) over the operating phases.

On the 130th day, there was a sealing problem in the solenoid valve responsible for releasing or restricting the passage of compressed air to the reactor. As a result, a large fraction of the biomass was washed out from the system during the reactor discharge step, since the biomass was distributed throughout the entire reactor volume due to continuous aeration. To avoid the development of filamentous bacteria again, the biomass that left the reactor by the natural disposal of the effluent, together with what remained, was stored for three days at 4 °C, until the operational problem was solved. From phase IV, by returning the iron ions to the influent stream, the amount of biomass in the reactor was reestablished, with TSS and VSS

contents around 8.52 and 5.5 g L⁻¹ (Figure A.3b), very close to those obtained in phase I (stable operation).

Values for the SVI, both for sedimentation within 5 (SVI₅) and 30 (SVI₃₀) minutes, are shown in Figure A.4a. In phase I, the ratio between SVI₃₀ IVL₅⁻¹ remained close to 1.0, with SVI₃₀ values between 40 and 60 mL g⁻¹. After the disturbance, with the reactor being dominated by filamentous bacteria, there was a slight decrease in the SVI₃₀ SVI₅⁻¹ ratio to values lower than 0.8, as five minutes were not enough for the sludge to settle completely. During this period, the SVI₃₀ increased to approximately 100 mL g⁻¹, a value similar to that commonly found for activated sludge, in the range of 80 to 120 mL g⁻¹ (SARMA; TAY, 2018). With the recovery of granular biomass in phase V, the quotient between SVI₃₀ SVI₅⁻¹ approached values close to 1.0.

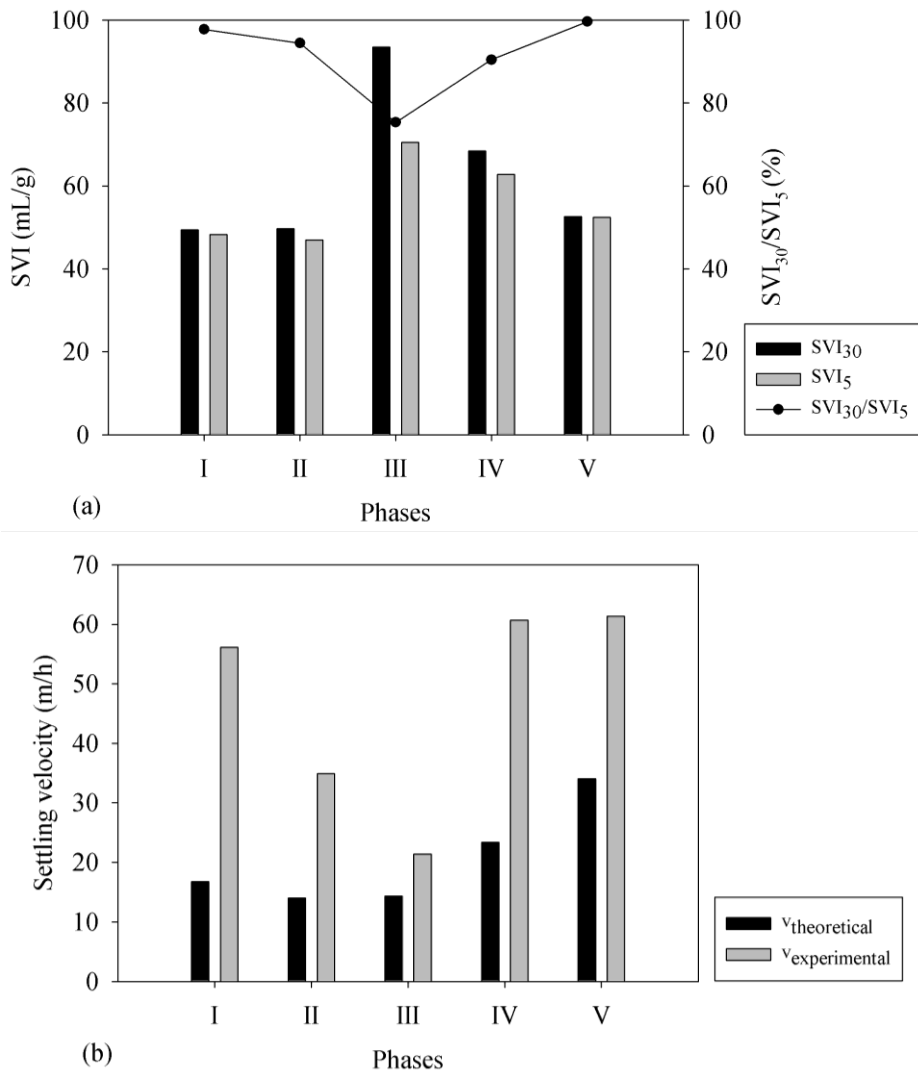


Figure A.4 - Granular biomass sludge volume index (a) and settling velocity (b).

Throughout the entire monitoring period of the SBR, the settling velocity obtained experimentally was observed to be higher than the theoretical one (Figure A.4b) and those reported for activated sludge (10 to 20 m h⁻¹) (NANCHARAI AH; KUMAR, 2018), even in the presence of filamentous organisms in the granules (phases II and IV). Throughout the recovery period, the velocity assessed experimentally increased again, reaching values between 50 and 80 m h⁻¹, close to those observed in phase I. The theoretical settling velocity was calculated by taking into account the water and granules density. From phase I to phase III, the granules showed very similar sedimentation velocities and close to that of activated sludge. This observation indicates that the theoretical settling velocity did not clearly illustrate the presence of filamentous organisms in the granular biomass, because these, as illustrated in Figure A.4a, adversely affected the biomass sedimentation.

Extracellular polymeric substances (EPS) are viscous materials produced by microbial cells which play an essential role in the formation and stability of aerobic granular sludge, with PN and PS representing the most important constituents (SHENG *et al.*, 2010). The sludge PN and PS contents were quantified, and the results are illustrated in Figure A.5. Both EPS components exhibited a similar trend during all phases of reactor operation. Moreover, PN content was always higher than PS content. It can be seen that the PN PS⁻¹ ratio decreased considerably in phase III as the difference between the concentration of proteins and polysaccharides decreased, indicating that protein production was more affected by the periods of disturbance. As previously reported, proteins play an important role in the stability of granules (LONG *et al.*, 2015). The addition of iron ions played a positive role in EPS production, mainly in PN content, as observed in phase IV. On the other hand, it has been reported that PS can form a strong and sticky structure, so the high PS content in granules may aid the formation and maintenance of a stable granular structure (CAI *et al.*, 2018). With the increased EPS content in the phase IV, the granules were able to restructure.

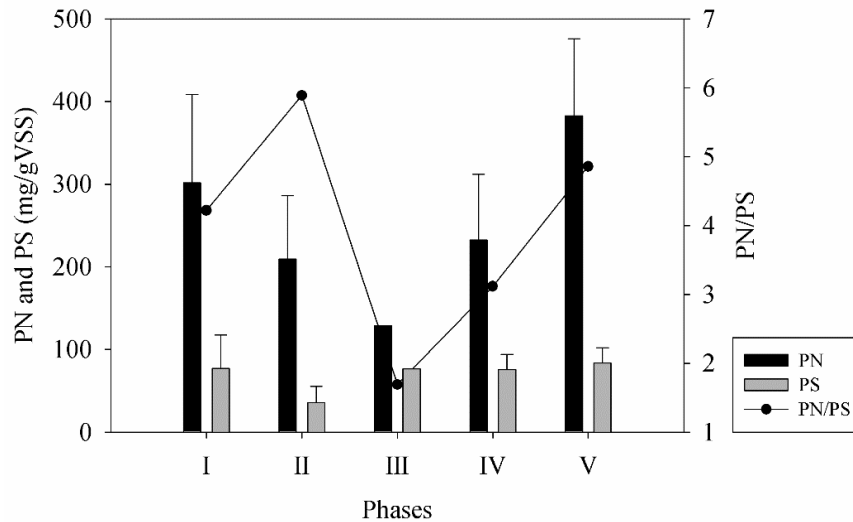


Figure A.5 - EPS content quantified as PN and PS over the entire experiment.

A.3.2 COD, P and N removal

The performance of the AGS reactor was evaluated in terms of organic matter and nutrient (N and P) removal. In the first 88 days of reactor monitoring, COD removal was higher than 90% (Figure A.6). Most of the incoming COD (around 89%) was removed in the anaerobic feeding period, mostly likely by PHA-producing organisms. After the system disturbance event designated as DI (phase II), COD removal decreased in the anaerobic phase, despite the presence of oxygen for the consumption of acetate (carbon source in the influent wastewater) by fast-growing heterotrophic bacteria (BASSIN *et al.*, 2019). The main issue affecting reactor operation was the growth of filamentous bacteria, which deteriorated the sludge settling properties, causing a massive washout of the biomass during the treated effluent discharge stage of the SBR. Because of the sludge loss, the food to microorganisms ($F M^{-1}$) ratio increased from $0.49 \text{ gCOD gVSS.d}^{-1}$ (phase I), a suitable value for stable granulation, as reported by WU *et al.* (2018), to $1.0 \text{ gCOD gVSS.d}^{-1}$ (phase II). However, biomass loss did not change the overall COD removal efficiency, which remained above 88%.

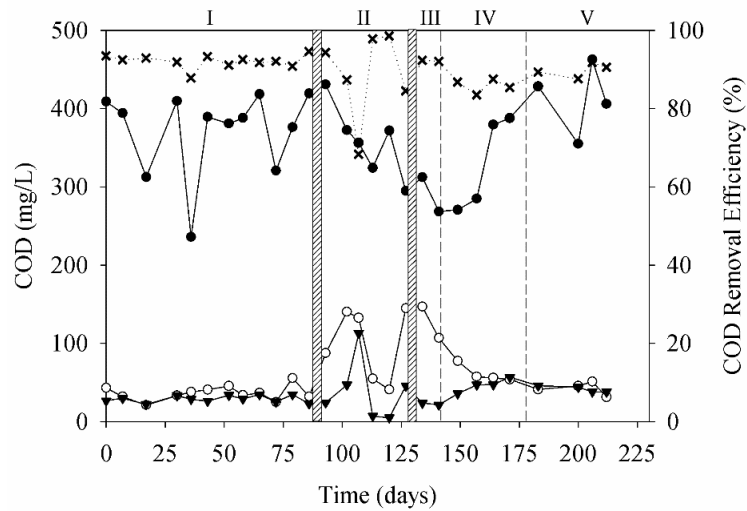


Figure A.6 - Performance of the AGS reactor over time in terms of COD removal. COD was analyzed at the influent (●), after the anaerobic feeding (○), and effluent (▼). The removal efficiency is represented by (×). The first and the second (from left to right) hatched bars represent the disturbance periods (DI and DII, respectively).

Under normal conditions, the reactor was operated under anaerobic-aerobic regime to favor the establishment of the enhanced biological phosphorus removal (EBPR) process through the development of PAOs. Thus, the release of phosphate in the anaerobic period was expected, followed by its consumption in the aerated phase. This results in the incorporation of phosphate into the sludge mass and, therefore, P removal from the system through the disposal of excess biomass. This profile was indeed observed in the stable operating period (phase I), during which the biomass-specific phosphate release reached values higher than 20 mgP gVSS⁻¹ (Figure A.7b).

However, when the filamentous bacteria developed after air leakage into the anaerobic feeding phase (period DI), the typical transformations of the EBPR process were impaired. The specific phosphate release in the anaerobic period decreased to only 3.7 mgP gVSS⁻¹ at the end of phase II. In the latter experimental phase, with the addition of iron ions to the influent, along with the nutrients (Solution 2), the influent phosphorus concentration was found to be lower (~7.0 mgP L⁻¹) than expected (15 mgP L⁻¹) based on the preparation of the synthetic wastewater (Figure A.7a). Therefore, it was hypothesized that phosphorus precipitation was occurring in the influent storage tank. Even though iron contributed to restore anaerobic phosphate release by enhancing PAO activity, PO₄³⁻ concentration after anaerobic feeding was still lower than that observed in phase I (Figure A.7). This could be

partially caused by the lower P COD⁻¹ ratio in the influent stream (SCHULER; JENKINS, 2003).

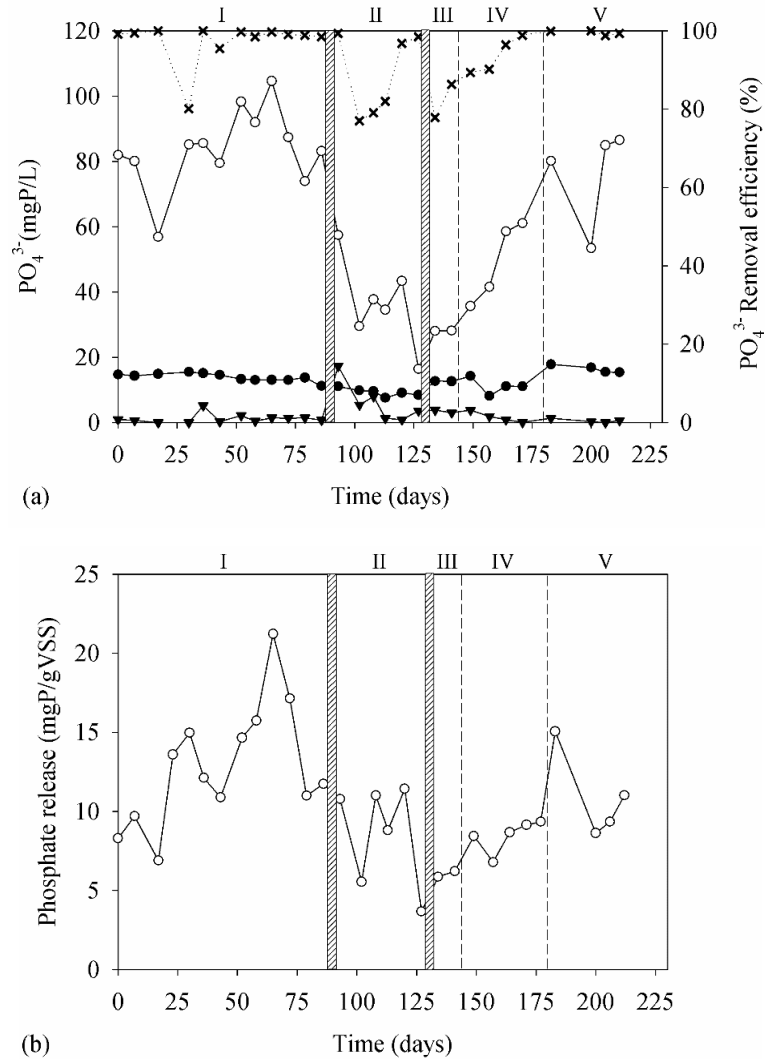


Figure A.7 - Phosphate removal achieved over AGS reactor operation (a) and phosphate release per VSS in the anaerobic feeding (b). Concentrations were analyzed at the influent (●), after the anaerobic feeding (○), and effluent (▼). The removal efficiency is represented by (×). The first and the second (from left to right) hatched bars represent the disturbance periods (DI and DII, respectively).

As a way to prevent this precipitation, iron started to be added to the tap water tank (phase IV). From this period onwards, a trend of recovery of the EBPR process was observed, and the specific phosphate release was less variable, with an average value of 8.5 mgP gVSS⁻¹ (for phase IV). In the last phase, after a decrease of phosphate released by biomass, the

average value was $9.7 \text{ mgP gVSS}^{-1}$ (Figure A.7b), indicating signs of recovery of the EBPR process. The ammonium concentration profiles at the different phases of the SBR operation are shown in Figure A.8a. It should be noted that ammonium levels after anaerobic feeding appear lower than in the influent due to the dilution of the influent stream with the liquid remaining in the reactor from the previous cycle (63% of the volume is retained, not exchanged from one SBR cycle to another), not due to removal by biological means.

Ammonium removal is associated with the nitrification process occurring in the aerated period of the SBR, and amounted to above 95% during most of the reactor operation, except after the disturbance events (DI and DII). During phase I, high ammonium removal efficiency was attained, with the average ammonium concentration in the influent and effluent of 54.5 and 1.7 mgN L^{-1} , respectively. However, ammonium removal dropped to 60% in phase II, immediately after DI. The deterioration of nitrification performance in phase II was likely caused by the significant biomass loss due to poor sludge settling conditions (as evidenced by SVI measurements, shown in Figure A.4a). Under these conditions, washout of slow-growing nitrifiers took place, whose activity takes time to be reestablished. In addition, COD was not completely removed in the anaerobic phase, so its presence in the aerated period stimulated the competition between fast-growing heterotrophic and nitrifying bacteria for DO and granule space within the oxygen-containing zone (BASSIN, 2018), with potential advantages for the first.

Once the granules recovery began within phase II, the increase in biomass content and the fact that organic matter was again completely taken up in the anaerobic period, the development of nitrifying bacteria was boosted. Consequently, ammonium removal efficiency reached 97% at the end of phase II, similar to that obtained during stable operation in phase I. After the second disturbance period (DII), ammonium removal efficiency dropped sharply again to around 65% in phase III. However, after 15 days under normal operation conditions, the nitrifying capacity was restored. Unlike this study, MOURA *et al.* (2018) reported that the performance of an AGS system in terms of organic matter and ammonium removal was not affected by biomass washout due to the presence of filamentous bacteria.

Nitrite and nitrate concentrations in the effluent were regularly analyzed to evaluate nitrogen conversions during the operating phases (Figure A.8b). As nitrite and nitrate are not present in the influent, these species are only shown for effluent samples. Nitrite concentrations close to zero were obtained during the stable phase (phase I), showing that complete nitrification was obtained. However, from phase II to V, nitrite build-up was

observed, with an average effluent concentration of 9.9 mgN L^{-1} . This result implies that, after biomass loss due to filamentous outgrowth resulting from the disturbance of anaerobic period by oxygen addition, partial nitrification was predominant. This result is associated with the selective NOB washout of the reactor, which, even with the return of stable conditions, no longer allowed complete nitrification to be obtained. The average nitrate concentration in the effluent during phase I was 3 mgN L^{-1} , falling to values between 0 and 1 mgN L^{-1} in the following phases.

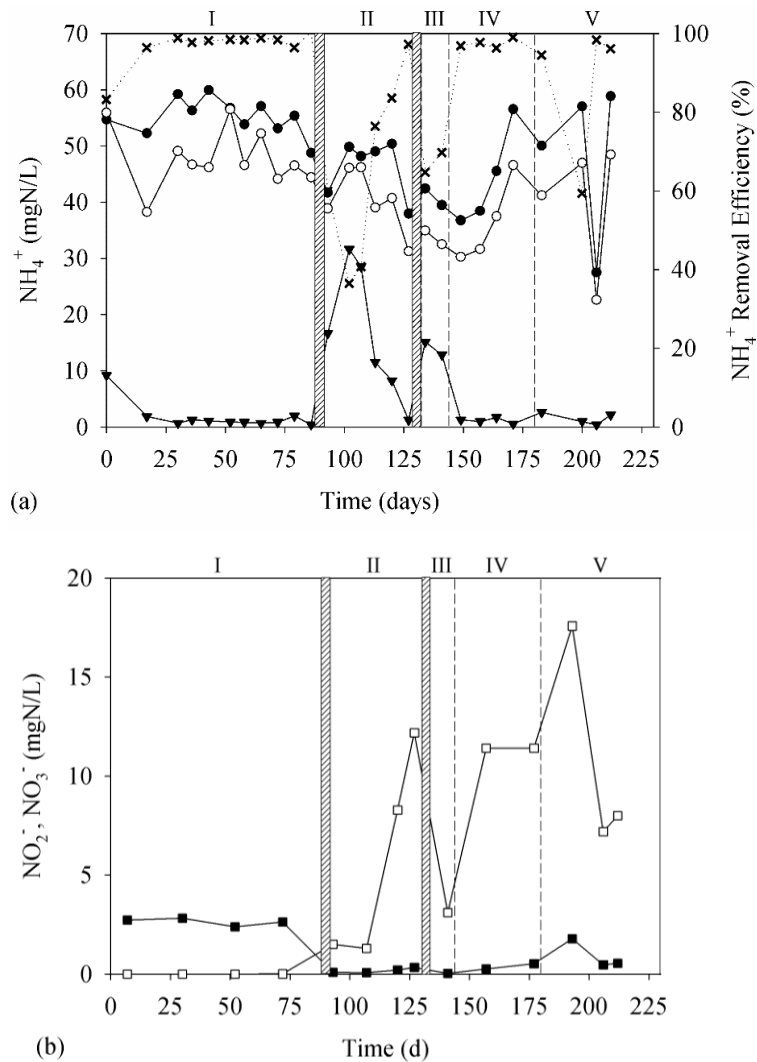


Figure A.8 - Nitrification process assessment over time. Nitrogen concentration was evaluated in terms of ammonium (a), nitrite, and nitrate (b). Their respective concentrations are represented by (●) influent NH_4^+ ; (○) NH_4^+ after anaerobic feeding, (▼) NH_4^+ effluent, and (×) NH_4^+ removal efficiency; Effluent (□) N-NO_2^- , (■) N-NO_3^- . The first and the second (from left to right) hatched bars represent the disturbance periods (DI and DII, respectively).

In addition to nitrification and denitrification processes, nitrogen compounds are removed by bacterial assimilation for growth purposes (anabolism) (Figure A.9). The contribution of assimilation (estimated by Equation A.1) is usually less important for total overall nitrogen removal. Nitrogen was mainly removed by denitrification within the anoxic zone of the granular biomass during the aerated period. The amount of nitrogen removed by denitrification decreased after the first disturbance (DI). It was only in phase IV that a slight recovery in denitrification levels was noticed. There was an exception to this behavior on day 193, when a significant amount of nitrite (17.5 mgN L^{-1}) was detected.

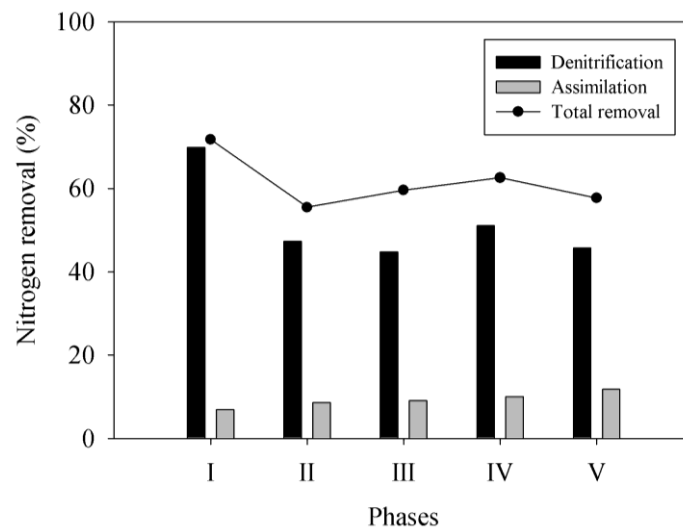


Figure A.9 - Nitrogen removal by assimilation and denitrification.

Cycle tests were carried out during the last phase to assess the dynamics of several compounds over the operating cycle (Figure A.10). Around 87% of the incoming COD was removed within the 60 min of anaerobic feeding period, remaining almost constant until the end of the cycle (180 min). This implies that the rest of the COD is not biodegradable. On the other hand, ammonium was oxidized within the first 80 min of the aerated period. Effluent nitrate and nitrite concentrations were 7.86 and 11.15 mgN L^{-1} , respectively. Therefore, taking into account the influent nitrogen concentration (as ammonium) and the nitrate and nitrite concentrations at the end of the cycle (no residual ammonium was detected), the nitrogen removal was calculated to be 75%. Regarding the EBPR process, it was observed a phosphate release of 110 mgP under anaerobic conditions. Moreover, P removal was almost complete (99.7%) within 70 min of aeration, characterizing a stable EBPR process. The specific ammonium, phosphate, and NO_x^- (denitrification) removal rates are shown in Table A.2.

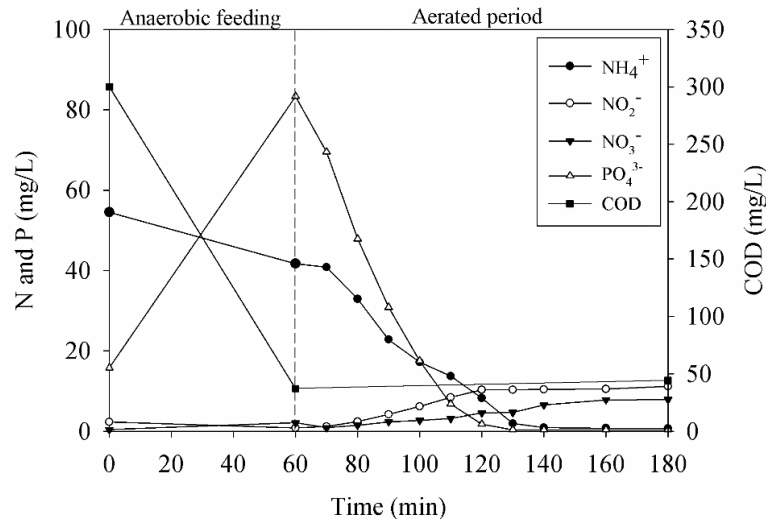


Figure A.10 - Cycle test conducted in the last phase (V). No measurement was carried out during the non-mixed anaerobic feeding period.

Table A.2 - Specific NH₄⁺, NO_x⁻ and PO₄³⁻ removal rates obtained in the cycle test carried out in stable conditions (phase V).

Specific removal rates	Values
NH ₄ ⁺	6.95 mgN gSSV ⁻¹ h ⁻¹
NO _x ⁻ (Denitrification)	5.31 mgN gSSV ⁻¹ h ⁻¹
PO ₄ ³⁻	14.59 mgP gSSV ⁻¹ h ⁻¹

A.4 Conclusion

The results of this study emphasized the need for keeping a stable and adequate operation of aerobic granular sludge systems. AGS depends on a true anaerobic period for the development of slow-growing organisms, such as PAO e GAO, while preventing rapid heterotrophic and filamentous outgrowth that occurs when oxygen becomes available during the anaerobic phase of the sequencing-batch reactor. The disturbance caused by the introduction of aeration during the phase that was designed to be anaerobic led to the proliferation of filamentous organisms, which impaired the biomass settling properties. Under these conditions, massive sludge washout and deterioration of effluent quality were observed, as organic matter but mainly nutrient (N and P) removal was adversely affected. During the

biomass loss periods, NOB were washed out of the reactor, leading to the accumulation of nitrite and partial nitrification. To suppress the growth of filamentous bacteria, iron addition proved to enhance granular stability. With the increase in EPS content, the granules were restructured, allowing restoring stable reactor operating conditions with good pollutants removal efficiency.