Microbial ecology of a sequencing batch reactor system with aerobic granular sludge for textile wastewater treatment

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Abstract

Due to the presence of color and consequent aromatic amines, textile industry wastewater represents one of the main sources of water pollution worldwide. In this sense, anaerobic-aerobic systems have been studied to overcome this scenario. This master thesis analyzed the mixed microbial culture present in aerobic granular sludge (AGS) sequencing batch reactors (SBRs), operated in anaerobic-aerobic cycles in different hydrodynamic regimes for the treatment of a synthetic textile wastewater, using a sizing agent (Emsize E1) as carbon source and Acid Red 14 as dye. Fluorescence *in situ* hybridization (FISH) allowed the identification of a specific group of bacteria, *Defluvicoccus vanus* related GAOs, which could be involved in amine biodegradation. It was also detected the importance of microbial culture diversity in granules stability. Furthermore, it was established the impact of sludge storage in community changes. It was also studied the effect of silver nanoparticles (AgNPs), at a concentration of 10 mg/L, in the granulation process through the analysis of the N-acyl-homoserine lactones (AHL) content, which is believed to be important for granulation. In the reactor with AgNPs, at the beginning of operation a delay in AHL production occurred, and it was detected that the granulation is related to an increase in AHL production and the same increase occurred when were registered biomass losses. This study contributed to a better knowledge of the microbial ecology of AGS for textile effluent treatment, which is important to improve the design and operation of these systems, towards mitigating this important source of pollution.

Keywords: Aerobic granular sludge; Textile wastewater; Wastewater microbiology; Aromatic amine biodegradation; Quorum sensing; Engineered nanoparticles.

Resumo

Devido à presença de cor e ao aparecimento de aminas aromáticas por degradação anaeróbia, os efluentes da indústria têxtil são uma das maiores fontes de poluição da água. Neste sentido, sistemas anaeróbios-aeróbios têm sido estudados por forma a alcançar uma solução sustentável para o tratamento desta água residual. Nesta tese de mestrado analisaram-se as comunidades presentes nos grânulos aeróbios em sistemas de reatores descontínuos sequenciais operados em ciclos anaeróbios-aeróbios em diferentes regimes hidrodinâmicos para o tratamento de um efluente de indústria têxtil sintético, usando Emsize E1 como fonte de carbono e Acid Red 14 como corante. *Fluorescence in situ hybridization* (FISH) permitiu a identificação de um grupo de bactérias, *Defluvicoccus vanus related GAOs* que podem estar envolvidos na biodegradação da amina. Demonstrou-se o impacto do armazenamento na comunidade. Por outro lado, estudou-se o efeito da presença de nanopartículas de prata (AgNPs), numa concentração de 10 mg/L no processo de granulação pela análise da concentração de N-acil-homoserina lactonas (AHL). No início da operação houve uma adaptação tardia por parte do reator alimentado com AgNPs imediatamente antes de se registar o aumento da concentração de AHL no início da granulação e detetou-se aumento na produção de AHL aquando da perda de biomassa. Este estudo contribuiu para um melhor conhecimento da microbiologia associada aos grânulos aeróbios para o tratamento de água residual de indústria têxtil, que é de extrema importância para o melhoramento do design do sistema e das condições de operação por forma a atenuar os problemas desta fonte de poluição.

Palavras-chave: Grânulos aeróbios; Águas residuais têxteis; Microbiologia das águas residuais; Biodegradação de aminas aromáticas; *Quorum sensing*; Nanopartículas.

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List of acronyms

AgNPs – Silver nanoparticles

AGS – Aerobic granular sludge

AHL – N-acyl-homoserine lactones

AIs – Autoinducers

CAS – Conventional activated sludge

COD – Chemical oxygen demand

CS – Conventional Sludge

Cy3 – Cyanine 3

DMSO – Dimethyl sulfoxide

EDTA – Ethylenediamine tetraacetic acid

ENPs – Engineered nanoparticles

EPS – Extracellular polysaccharides

FAS – Flocculent activated sludge

FISH – Fluorescence in situ hybridization

FITC – Fluorescein isothiocyanate

GAOs – Glycogen-accumulating organisms

OD – Optical density

OD_{600} – Optical density at 600 nm

OD_{615} – Optical density at 615 nm

ODHL – (N-(3-oxo-dodecanoyl)-L-homoserine lactone

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

QS – Quorum sensing

SBR – Sequencing batch reactor

SRT – Sludge retention time

SVI_5 – Sludge volume index at 5 min

SVI_{30} – Sludge volume index at 30 min

TSS – Total suspended solids

WWTP – Wastewater treatment plant

UPLC – Ultra performance liquid chromatography

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1 Introduction

1.1 Problem context

The need to safeguard water resources has been recognized worldwide as a priority in many industrial sectors, therefore, an effort has been done in the scientific community to search for new adequate wastewater treatments and to develop potential environmental friendly and economically attractive technologies [1]. Regarding the increasing demand for textile products, textile industry has become one of the main sources of water pollution. In particular, the wastewater from the textile finishing industry represents an environmental problem and also, a public health concern, mainly due to the presence of recalcitrant dyes and high organic loads [2]. Moreover, this industry is a heavy consumer of potable water, which consequently leads to a decrease in water quality, particularly in countries with water scarcity-related problems [1].

Color in textile industry effluents, promoted by the presence of dyes, remains as a serious concern. The environmental problem caused by undesirable colored wastewater it is not only due to a noticeable issue, but it can also interfere with the equilibrium of the receiving water media ecosystem [3].

Dyes can be classified according to the context of their application or their chemical structure [4]. Azo dyes are a potentially toxic class of dyes that are resistant to aerobic degradation, even so, they represent the most widely chemical class of synthetic dyes used in the textile industry [5]. The first step of the biological degradation of azo dyes has been described as the anaerobic azo bond cleavage, leading to color removal, with the consequent formation of aromatic amines that are not further degraded anaerobically [2]. Without the proper treatment, the textile wastewater represents a wide environmental problem. Nevertheless, the removal of color has been an intensively addressed challenge over the years [6].

In order to achieve not only azo dye decolorization, but also the aerobic mineralization of the resulting aromatic amines, two-stage anaerobic/aerobic reactors based on flocculent activated sludge have been proposed [5].

Regarding the operational issues of conventional flocculent activated sludge systems, namely, settling problems, aerobic granular sludge (AGS) in anaerobic-aerobic sequencing batch reactor (SBR) systems has been considered as a good alternative to treat textile wastewaters [2]. In fact, AGS presents excellent settling properties, is less sensitive to high organic loads and toxic compounds and, under specific conditions, the granular structures have aerobic and anoxic-anaerobic zones co-existing in the same reactor [7].

The evaluation of AGS microbial community diversity and dynamics plays an important role in these SBR systems, as well as the evaluation of its azo dye degradation capability [2]. In order to support the potential application of AGS technology, research on granulation and long-term operation has been done using different bioreactor configurations. However, it is believed that the

process of granulation is highly dependent on cell-to-cell interactions, such as quorum sensing (QS) communication [8]. Furthermore, QS has become a new approach to investigate the microbial attachment and biofilm formation in mixed cultures. N-acyl-homoserine lactones (AHL) are chemical molecules produced by Gram-negative bacteria that are implicated in QS. Even more, they have been described as important in pure culture interactions and one of the most studied groups of auto inducers (AIs) [9].

The study of microbial activity and bacterial growth inside the aerobic granules has been established as a priority since microbial ecology is related to functional outcomes and the distribution of the bacterial types in a population determines the ecosystem processes [10]. For the case of textile wastewater treatment, the difficulty in achieving azo dye and aromatic amine mineralization has been described as highly associated to the lack of adequate microbial groups [3]. Therefore, it is particularly relevant to identify the microbial populations that are responsible for the mineralization of azo dye reduction products under aerobic conditions. In fact, understanding biomass adaptation to changes in bioreactor operating conditions is considered as a good approach to evaluate textile wastewater treatment performance.

1.2 Dissertation objectives and outline

The goal of the current work was to evaluate the impact of textile wastewater on AGS microbial community structures during aerobic granule formation and long-term AGS operation in terms of microbial population diversity and dynamics. In addition, the identification of organisms capable of biodegrade aromatic amines it has also been attempted. For that, key biomass samples were selected from 5 different operational periods and were characterized using fluorescence *in situ* hybridization (FISH).

The importance of quorum sensing in biofilm formation has been previously described [8]. Thus, the communication between the cells and their surroundings during granulation was also evaluated through quorum sensing detection and quantification of signaling molecules, which were correlated to granule stability and SBR performance. It was also assessed the impact of engineered nanoparticles (ENPs) in granulation process.

The dissertation is organized in 6 chapters. After a detailed description of all the recent and more important developments regarding the textile industry wastewater treatment theme, the methods used in each part of the laboratory work are reported, with a first description of the different SBR operations performed since 2013 by the lab group.

The results are graphically presented after the methods, in parallel with some observations. And finally, the conclusions of all the work and future perspectives are explained in a specific chapter.

2 State-of-the-art

2.1 Textile industry wastewater

With the increasing demand for textile products, colored textile wastewater became one of the main sources of pollution worldwide.

The textile industry consumes large quantities of water, and depending on the type of fabrics produced, the amount vary between 0.08 m^3 and 0.15 m^3 per 1 Kg of fabrics [11]. In addition, it produces huge volumes of wastewater (between 3800 and 7600 million m^3 per day) rich in non-biodegradable components [12].

It has been described that the dyeing processes are responsible for releasing around 90% of all the chemicals used in the textile industry. On the other hand, dye fixation levels between 50% and 90% have been reported [12].

Textile industries are characterized depending on the type of fiber used. However, the majority of the fabrics are manufactured from cotton lines and from wool. Textile industry effluents usually have high levels of chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total suspended solids (TSS). In addition, textile industry wastewater has an intense color and it is also associated with high salt concentrations [12]. Azo dyes are one of the most broadly used dye classes. However, they are highly persistent and recalcitrant [2].

The presence of recalcitrant dyes and high organic loads are the main issues of textile wastewaters. Therefore, these parameters need to be strictly controlled [2]. Conventional aerobic bioprocesses generally fail to treat textile wastewater due to its non-biodegradable nature. Also, the color is not removed aerobically and can make a river inhabitable to the majority of aquatic plants and animals [12]. On the other hand, under anaerobic conditions the color is removed but the azo dyes are transformed into aromatic amines that can be more toxic than the original dyes.

2.1.1 Nanoparticles

In association with the increasing production of textiles, new textile materials have emerged. The textile industry has recognized the advantages of applying engineered nanoparticles (ENPs) onto textiles. ENPs have antibacterial/antimicrobial properties, and they can confer water resistance and protection to the fabrics. For this reason, also cosmetics, food industry, coatings, pharmaceutical industry and microelectronics have interest in their usage [13].

Silver NPs (AgNPs) have gain textile industry's interest due to their antimicrobial and antibacterial effects, which lead to odor elimination in, for instance, athlete's clothes, jogging outfits, and medical textiles. However, negative aspects are associated to the increasing introduction of ENPs in industrial processes. In fact, their release through the environment implies an environmental risk. Despite the textile industry, also large hospitals and hotel laundries contribute to ENP release in the environment. Nevertheless, several studies reported the toxic

effects of ENPs in living organisms and also DNA damage. Despite their excellent properties, the commercial application ENPs should be carried out carefully [13].

A proper handling and disposal of ENPs is needed. Thus, appropriate analytical methods for ENP monitoring are also required. The main issue is related to the ability of ENPs to interfere with the microorganisms present in wastewater treatment plants [13]. In this context, further research should be performed.

2.1.2 Azo dye and aromatic amine degradation

The following scheme (**Figure 2.1**) presents an effective metabolism for azo dye degradation, only achieved with a combination of anaerobic and aerobic processes.

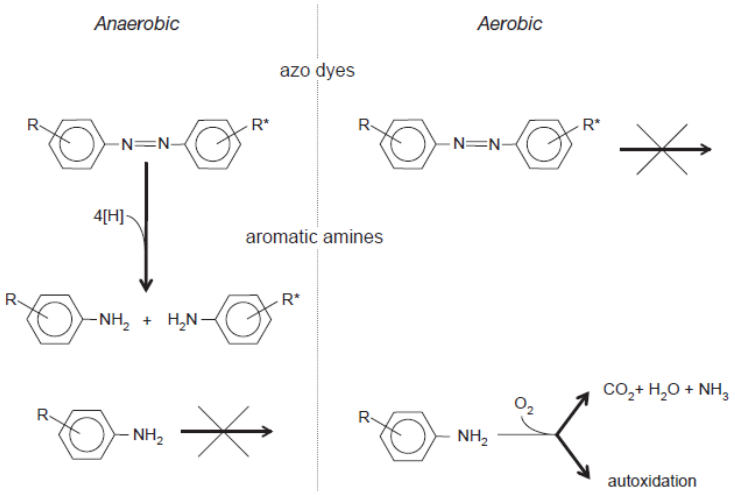


Figure 2.1: General overview of the azo dyes and aromatic amines degradation under anaerobic-aerobic treatment [5].

Since 1980s many bacterial cultures have been reported as capable to remove color derived from different azo dyes. For instance, strains of *Aeromonas hydrophila*, *Bacillus subtilis* and *Pseudomonas cepacia*. Under anaerobic conditions, bacteria, via azoreductases, use azo-bonds as the electron acceptor, reducing it into aromatic amines [5].

The color removal in azo dyes occurs when the azo bond is cleaved, since the resulting amines are mostly colorless. The azo bond cleavage –N=N– involves a transfer of four-electrons, which proceeds through two stages at the azo linkage. In each stage two electrons are transferred to the azo dye, which acts as a final electron acceptor [4].

Beyond color removal, a growing concern regarding the release of chemical compounds into the environment has been noticed in the last decades. In this context, aromatic amines have been reported as being one of the main issues. Scientific developments and efforts in regulation aspects have been done. However, the constant emergence of new products presses the market to find solutions to reduce their environmental impact. Pinheiro et al. (2004) [14] described the

main sources of aromatic amines as being derived from sectors such as oil refinery, synthetic polymers, dyes, adhesives and rubbers, pharmaceuticals, pesticides and explosives, also it is described their potential relation to cancer development.

Since some amines produced by azo dye reduction can be toxic and have been implicated in cancer development in humans [14] it is highly important to monitor these azo dye intermediates. Therefore, an effort has been done to develop analytical methods to detect aromatic amines in water samples [14]. However, according to Zee & Villaverde (2005) [5] new insights are needed, particularly, regarding the detection of aromatic amines, the degree of mineralization and the characteristics of the dye residues.

The main issue is the generally low aromatic amine biodegradability. However, Ekici et al. (2001) [15], quoted by Pinheiro et al (2004) [14] mentioned that some aromatic amines have been found to be biodegraded by activated sludge with better results under aerobic conditions. Furthermore, a review paper summarized bacterial degradation of monocyclic aromatic amines [16]. Many bacteria have been isolated and it was discovered their ability to mineralize monocyclic aromatic amines.

Indeed, over the years, several mechanisms have been proposed for monocyclic aromatic amines mineralization. Bacterial aerobic degradation of monocyclic aromatic amines may be initiated via one of the following mechanisms: a) aromatic ring cleavage catalyzed by a dioxygenase; b) aromatic amine dioxygenation; c) aromatic amine deamination; d) hydroxylation of aromatic amine; e) aromatic amine co-ligase mediated activation to coenzyme A (CoA) thioesters and f) dehalogenation of chlorinated aromatic amines [16].

Nevertheless, it remains as uncertain the capacity for aromatic amines breakdown by a mixed microbial community, and more research should be carried out to investigate the conditions that promote this process in wastewater treatment systems.

2.1.3 Biological textile wastewater treatment technologies

Over the last decades it has been a challenge to find a reliable solution for textile wastewater treatment. Therefore, many studies have been published in this regard [2], [6], [3]. Biological and non-biological decolorization have been broadly described in literature.

In 2007, Santos et al. [4], reviewed the current technologies for decolorization purposes of textile wastewater. In 1990s several physical and chemical methods of dye removal were described and they are still available for textile wastewater treatment [4], such as adsorption by activated carbon, coagulation and sedimentation, bleaching with chlorine or ozone and reverse osmosis. However, these methods are characterized by high costs. Biological treatments have lower operating costs and better chances of progress since chemical processes are coupled to disposable problems and with environmental issues [6].

Studies were published in the last years regarding not only color removal, but also aromatic amine mineralization [4], [16]. Regarding biological degradation, both bacteria and fungi have an

important role and their capacity has been described as related with enzyme and exo-enzyme production. Moreover, it was described that the addition of redox mediator compounds could enhance decolorization. The chosen method is also highly dependent on wastewater characteristics, not only the class and concentration of the dyes present, but also the water properties such as pH, salinity and presence of toxic compounds.

2.1.3.1 Activated sludge systems

Conventional activated sludge (CAS) systems have been used for years as the preferred biological wastewater treatment and they are still the most used systems in order to remove organic carbon and nutrients [7]. However, because of their drawbacks, for instance, poor biomass settling properties, treatment facilities require a large area [17]. In this context, new research has been done, particularly with focus on trying to find the new generation of wastewater treatment processes, not only in textile wastewater treatment but in general wastewater treatment.

Anaerobic-aerobic sequencing batch reactors (SBRs) have been largely developed over the past decade. SBRs with flocculent activated sludge have been proposed for textile wastewater treatment. In Zee & Villaverde (2005) review [5], a broad analysis was performed evaluating the published bioreactor studies for industrial wastewater treatment.

Recently in the early 21th century, research papers presented successful decolorization under sequenced aerobic-anaerobic conditions. Lourenço et al. (2000) [6], developed an experiment with a SBR system for treating synthetic wastewater describing levels of COD removal around 80%, from which 30% were removed under anaerobic conditions, and also decolorization levels of 90% were reached after 40/50 days of acclimatization. However, despite the notable success in the anaerobic decolorization step, the attempt to obtaining a microbial community capable of mineralizing aromatic amines was not successful.

2.1.3.2 Aerobic granular sludge (AGS) systems

Aerobic granular sludge (AGS) can effectively grow under SBR operational conditions and has been implemented in several domestic and industrial wastewater treatment plants [2].

Due to the need of a first anaerobic step for color removal and a second one for aromatic amine mineralization under aerobic conditions, the granules' capacity of having both aerobic and anaerobic zones has been proposed as a solution for textile wastewater treatment. In addition, the fact that AGS can be operated at high sludge retention times (SRT), allows the growth of a more diverse microbial community including slow-growing populations known to degrade recalcitrant compounds. Furthermore, the resistance of aerobic granules to high organic loads and toxic compounds and also their stability can be highly advantageous for textile wastewater treatment [2].

Beun et al. (2002) [17], described AGS cultivation within a sequencing batch airlift reactor. The granulation occurred in a week after inoculation. Based on differences in settling velocities, granules were selected against flocs.

More recently in 2013, new significant advances were described in the AGS theme [7]. A team was responsible to implement a pilot plant operating with total success. Therefore, it was a very important advance for the technology regarding the lower use of energy and chemicals reported, even more, the lower investment and operational costs. This Nereda® technology was firstly developed in Delft University of Technology (**Figure 2.2**). It consists of a SBR system with cycles having three operational steps. In the feeding step, the wastewater is displaced in the bottom of the reactor while the effluent is drawn by the top. After the feeding step, the aeration starts and promotes the biological processes. Underneath the aerobic layer of the granules, the inner layers are anoxic due to mass transfer limitation of the oxygen and to its consumption by the aerobic organisms situated at the granules' surface. Ammonia oxidation in the external layers produces nitrate and/or nitrite, which are diffused to the inner layers where they can be denitrified. Therefore, AGS promotes efficient nutrient removal without the need of having different structures within the same reactor to obtain oxygen rich and oxygen depleted zones. The last step of the SBR cycle consists on a rapid settling phase to enhance granule selection against flocs.

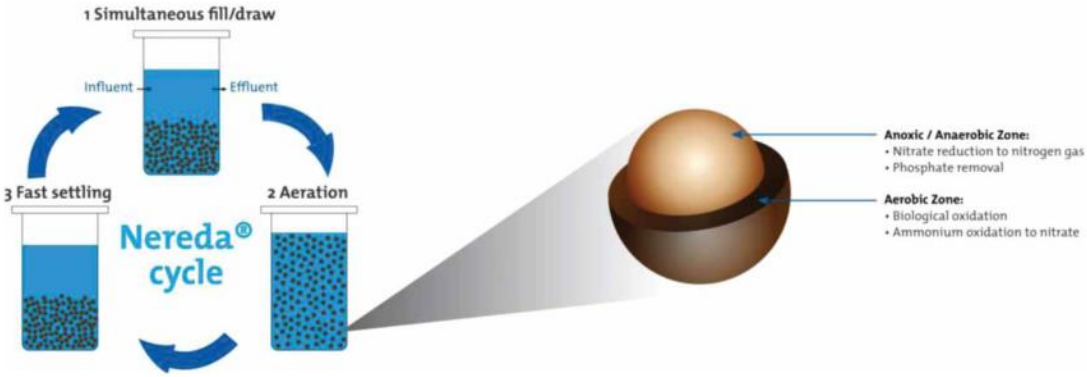


Figure 2.2: Schematic view of the Nereda® cycle [7].

This is a promising technology for textile industry wastewater treatment since, both aerobic and anaerobic zones are essential to achieve a proper dye degradation and further amine mineralization in parallel with a good COD removal efficiency [2], [3].

Regarding textile industry wastewater, despite the described success in the anaerobic decolorization step, the difficulty in amines removal remains an issue in the aerobic stage, probably due to inadequate microbial population selection.

2.2 Microbial ecology in wastewater treatment

2.2.1 Microbial community characterization techniques

Biological wastewater treatment processes are dependent on microbial organisms interaction and evolution of the community profile in adaptation to the operating conditions and wastewater characteristics. Specific and important processes regarding compounds removal rely on distinct microorganisms thus, their knowledge is essential for optimizing the design and operation of these systems [18].

Traditionally, microbial analysis was carried out by isolation. However, isolation approaches are very limiting, since approximately only 1% of the bacteria are cultivable [19]. Some culture-independent techniques, including polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), cloning and fluorescence *in situ* hybridization (FISH), have been used for decades to describe the microbial communities in mixed cultures [18], [20]. After the initial application of these approaches, the rise of the *omics* era has resulted in a turning point in studying phylogenetic and functional diversity of wastewater treatment systems [18].

Figure 2.3 represents the main molecular approaches used to study microbial community diversity in wastewater treatment sludge.

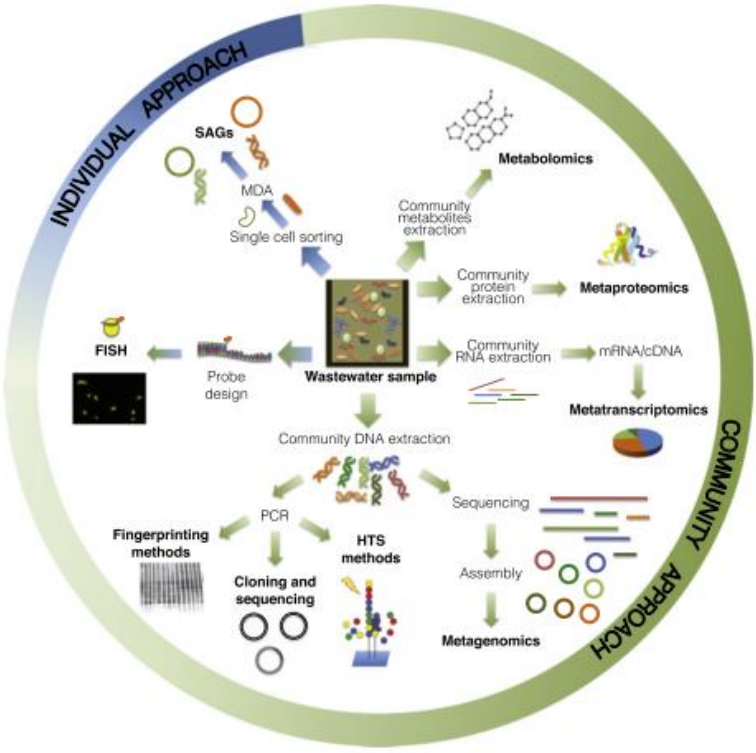


Figure 2.3: The community approach comprises those PCR- dependent methodologies for the analysis of the 16S rRNA gene, such as fingerprinting techniques, cloning and high-throughput sequencing (HTS), as well as PCR independent techniques for the analysis of DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics) and metabolites (metabolomics). The individual approach includes the analysis of single cell genomes (SAGs) through cell sorting and multiple displacement amplification (MDA). Fluorescence in situ hybridization (FISH) offers the possibility to perform studies both at the individual and community level [18].

2.2.1.1 Polymerase chain reaction

PCR techniques revolutionized the study of microbial diversity previously constrained by the inability to grow the majority of microorganisms in culture.

However, the well-known amplification bias and limitations in primer development could restrict the quantitative information that could be available [18].

2.2.1.2 Denaturing gradient gel electrophoresis (DGGE)

Since the discovery of DGGE that it has been widely used to study the microbial ecology of different activated sludge plants [21]. It also allows the simultaneous analysis of multiple samples, making it possible to follow community changes over time. However, for accurate results, the samples must be run in a single gel. Moreover, this technique is also dependent on a PCR step, with the constraints described above, and the sensitivity of the method is limited to a few bands (presumably corresponding to the different elements of the community) being detectable in the gel. Finally, this technique is also in disuse due to its high experimental burden, not only to ensure reproducibility of the DGGE band profiles, but also because for phylogenetic identification of the community members -i.e. bands- subsequent cloning and sequencing is required.

2.2.1.3 Fluorescence *in situ* hybridization (FISH)

FISH is a rapid and simple method and it has the advantage of being a culture-independent and PCR-independent technique which overcomes the limitations of the methods presented above. In addition, it can be used to detect microorganisms within complex samples such as flocs or granules, and simultaneously obtain information about their spatial distribution in the aggregates. Furthermore, being a non-destructive technique, FISH allows the observation of the morphologies of the microbial groups. FISH uses oligonucleotide probes that target ribosomal RNA (rRNA) that are group specific, which allows the identification of communities, providing also quantitative information with a confocal microscope [18], [22]. However, the hybridization only occurs in active cells leading to misleading results since, it is possible to have a bacterial group present and not active at that moment. In addition, the probes used in FISH are selected by the researcher and they are specific to one type of organism or group of organism therefore, the choice could narrow the analyses.

2.2.1.4 Next generation sequencing methods

Approaches including metagenomics and metatranscriptomics have been applied to massively evaluate the microbial community. The recent emergence of next generation sequencing (NGS) techniques such as Illumina and 454-pyrosequencing brought the identification of microorganisms to the next level. Despite the good coverage of this method for microbial community assessment, the relative read proportion may not correspond to the actual organism abundance, since the number of copies of the marker gene (most commonly the 16S rRNA gene) differs from organism to organism. [18].

Most of the published studies used more than one technique to evaluate the microbial community of biological wastewater systems.

2.2.2 Core community in wastewater treatment plants

A recent published work identified a core community of microorganisms present in wastewater systems [23]. The broad study evaluated the microbial community in 13 Danish wastewater treatment plants by sequencing the 16s rRNA gene through Illumina NGS and found a core of 63 abundant genus-level operational taxonomic units (OTUs), which represented 68% of the total reads. In addition, other studies referred the same genera in samples from China and the USA [24], which suggests that there could be a relationship between the plants around the world [23].

Figure 2.4 showed the results of the study and it allowed the identification of *Alpha- Beta-* and *Gammaproteobacteria* within the most abundant communities, and also revealed the presence of High G-C content Gram-positive bacteria and *Flavobacteria.* Considering these findings, for the present study, FISH was carried out with probes chosen to cover the most abundant organisms revealed in **Figure 2.4** in order to characterize the microbial community profile in the different reactor conditions assessed. In section 3.2.2 the probes used are specified.

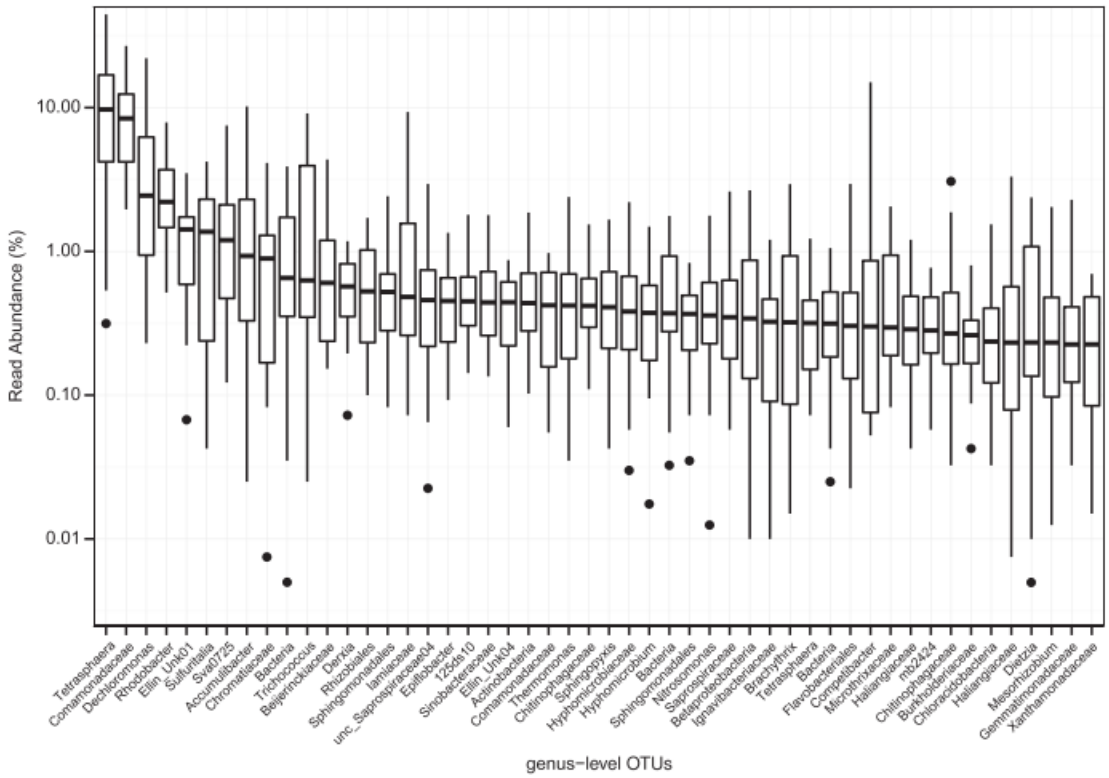


Figure 2.4: Graphic shown the abundance of the top 50 genus-level OTUs (by median) detected in 13 Danish wastewater treatment plants. The upper and lower bounds of boxes denote the 25th and 75th percentiles and the lines denote the max and min values registered. The dots correspond to outliers [23].

2.2.3 Microbial diversity in aerobic granular sludge (AGS)

Due to granules morphology, in aerobic granular sludge systems, different *Bacteria* has been identified. Nitrifiers are located in the in the granules surface and denitrifiers and the growth of phosphate accumulating organisms (PAOs) are associated to the inner layers [10].

FISH analyses were performed in sliced granules and it was demonstrated the typical spatial distribution of nitrifiers in the outer layer as well as PAOs, and glycogen accumulating organisms (GAOs) in the outer and inner layers. The study was developed using probes for most *Accumulibacter*, some *Competibacter*, *Nitrospira* like organisms, *Nitrobacter* and for all ammonia oxidizing *Bacteria* (AOB)[10].

Combining both AOB and anaerobic ammonia-oxidizing bacteria (anammox) has been described as an attractive option for nitrogen removal. Therefore, a work was developed in order to determine the abundance, distribution and activity of AOB and anammox. The results demonstrated that AOB abundance measured using quantitative FISH was significantly higher in flocs than in granules and anammox abundance was significantly greater in granules [25].

In other hand, it was shown that sulfate reducing bacteria (SRB) grew in granules in order to protect themselves for oxygen exposure and has been described as being present in aerobic WWTP [26]. Therefore, studies were released regarding the enhance of denitrification in granules. For instance, it was exploit a new granulation strategy in a denitrifying sulfur conversion-associated enhanced biological phosphorus removal and the results suggested that SRB and sulfur oxidizing bacteria (SOB) were associated to an improve of the phosphorus removal efficiency [27]. SOB were also tested for autotrophic denitrification and FISH technique revealed that granules were enriched with SOB contributing to autotrophic denitrification. Furthermore, 16s rRNA analysis showed diverse autotrophic denitrification related genera, namely *Thiobacillus*, *Sulfurimonas* and *Arcobacter* and no heterotrophic denitrification related were detected [28].

2.3 Cell communication: Quorum sensing

Generally, it is believed that the ability of microbial attachment is based on quorum sensing (QS). Up to now, experimental studies in this field were majorly developed in medicine and food chemistry and have a focus on pure cultures [8], [9]. However, recently researchers are also investigating QS effects on biofilm formation in mixed culture systems [9].

QS is a way of cell-to-cell communication. The regulation of gene expression occurs in response to changes in cell density by increasing the production and release of chemical signaling molecules called autoinducers (AI). When the bacteria identify a minimal threshold stimulatory concentration of an AI, an alteration of the gene expression occurs. Gram-positive and Gram-negative bacteria use QS to regulate processes such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation. In addition, it has been reported that cell-to-cell communication occurs either within the same species or between species [29]. Many Gram-negative bacteria produce N-acyl-homoserine lactones (AHL) and Gram-positive bacteria use processed oligo-peptides to communicate. Particularly AHL are the most studied group of signaling molecules and also play a role in gene expression regulation [30]. The increased transcription of certain genes leads to different phenotypes, according to Ravn et al. (2001) [30], probably the most known event is the release of bioluminescence in *Vibrio fischeri* regulated by an AHL. The effect of AHL has been well characterized regarding microbial attachment and biofilm formation [9]. Studies have been published considering the identification of specific AHL in biofilm regulation in pure cultures [9]. And more interestingly, the AHL effect in AGS formation has been examined [31], [8], [9].

In sections 2.3.2 and 2.3.3 are described some advances published regarding AHL importance in both, pure cultures and granules.

2.3.1 N-acyl-homoserine lactone detection by reporter strains

Chemical analyses have been done using an ultra-performance liquid chromatography (UPLC) to detect AHL molecules. However, this method requires ethyl acetate addition before analysis. The use of reporter strains to detect the presence of signaling molecules in extracts has been proposed as a viable alternative. Manipulated bacterial strains, including *Agrobacterium tumefaciens* and *Chromobacterium violaceum,* produce a phenotype only detected in the presence of AHL [28], [30].

The production of a blue color in the presence of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) is a widely used screening technique [8], [9], [33]. The molecular mechanism starts with X-Gal hydrolysis by the protein β-galactosidase and consequent formation of 5-bromo-4-chloro-indoxyl that oxidizes and spontaneously dimerizes to a blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo, which results in blue colonies. Colonies from genetic engineered strains which only express a functional β-galactosidase under specific conditions could be distinguished from others that were not submitted to the same conditions by their blue color. In addition, the

method could be used to distinguish between a successful cloning product from an unsuccessful one.

It was in 2002 that Farrand et al. (2002) [34] showed the utility of the QS system of *A. tumefaciens*. *A. tumefaciens* induces tumorous growths on susceptible host plants mainly due to the transfer of Ti plasmids (**Figure 2.5**), which lead to a certain genes expression and consequent tumor phenotype. These plasmids encode for two independent transfer systems; one, called *vir*, is responsible for processing and transferring the T-region from the bacteria to host plant cells. The second, called *tra*, confers transfer of the entire Ti plasmid from the host donor bacterium to a recipient bacterium by conjugation.

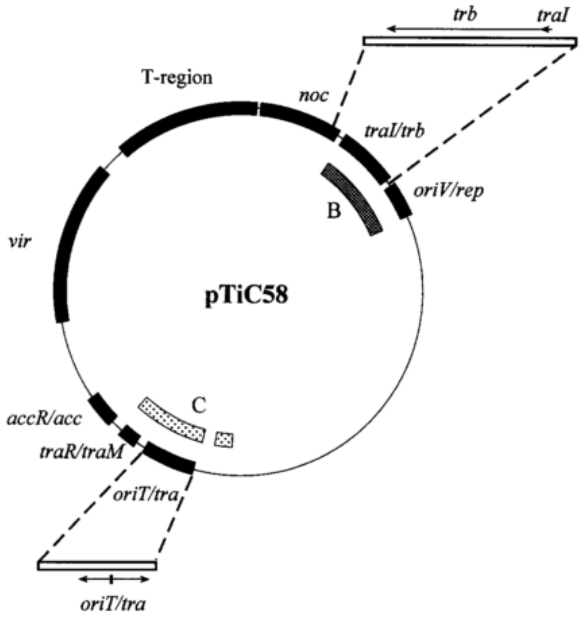


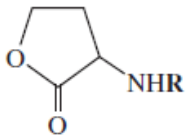
Figure 2.5: Physicogenetic map of the pTiC58 Ti plasmid. These elements are divided into functional segments including: *oriV/rep*, replication; T-region; *vir* region; opine catabolism (*noc*, nopaline catabolism and *acc,* agrocinopine catabolism); *tral/trb* and *oriT/tra*, the conjugal transfer (*tra*) regulon. The tra regulon is composed of three operons, *tral-trb* that encodes production of the AHL quormone and also the type IV secretion apparatus and two *tra* operons, *traAFB* and *traCDG* which are oriented divergently from an interoperonic region containing the *oriT* site. The shaded arcs inside the map represent two of the four regions that are strongly conserved between many Ti plasmid types [34].

A. tumefaciens NTL4 (pZLR4) detector strain lacks a Ti plasmid and is described as nonpathogenic. The strain also lacks *traI* and does not produce any detectable levels of AHL. This strain holds a plasmid, pZLR4, with *traG* fused with *lacZ* and *traR*, known to respond to low concentrations of AHL. Thus, in the presence of AHL the production of TraR protein occurs, which allows the activation of *traG* gene. However, due to their mutation, the production of β-galactosidase is expressed forming blue colonies in X-Gal containing medium, whereas white colonies appear in the absence of active signal.

It is relevant to mention that each reporter strain has its own detectable levels for different AHL molecules. These levels are highly dependent on the branched chain of the AHL molecules thus, the higher the carbon number the less the concentration required for the maximum activation of the reporter gene [33].

Furthermore, Singh & Greenstein (2011) [33] applied the knowledge of the reporter strains and developed a sensitive, rapid and simple approach to detect AHL compounds. An extended experiment was performed and validated in both the 96-well and 384-well plate formats.

In addition, four known synthetic AHL molecules were also found as potent inducers of the reporter gene, and their activities depended upon the length and the nature of the substitution at the C3 position of the molecules (**Figure 2.6**). Besides the fact that it was the first study suggesting a prevalence of AHL compounds found in natural extracts, it was mentioned as a new approach to deal with QS based investigation, since it was proposed a microtiter plate method to measure the AHL content without using chemical analysis. However, further research needs to be done regarding these findings.



HSL type	R	Active at
Butanamide	$CO(CH_2)_2CH_3$	500 nM
Octanamide	$CO(CH_2)_6CH_3$	0.15 nM
Hexanamide	$COCH_2CO(CH_2)_2CH_3$	0.50 nM
Tetradecanamide	$CO(CH_2)_{12}CH_3$	15 nM
NT1 filtrate	contains octanamide	$10^{-6 \text{ to } -7}$ diln

Figure 2.6: Known activities of some AHL [33].

2.3.2 Quorum sensing detection in pure cultures

The implications and mechanisms of QS in pure cultures have been studied for a long time [9].

Regarding the food field, a wide-range study was developed to screen 148 Enterobacteriaceae isolated strains [30]. Furthermore, it represents the first attempt to describe a simple way to analyze QS signaling molecules. The methodology used consisted in screening AHL production using agar well-diffusion assays. Those assays were prepared diluting reporter strain culture in the last exponential phase in new solid fresh media. The advantage of this kind of approach is that it does not require sterile supernatants or AHL extracts, representing a fast method. AHL extraction supernatants or synthetic AHL were applied into handmade punched wells in the solidified agar. Later, the plates were incubated at stable temperature (suitable for the strain in use), then the diameter of the surrounding zone was measured for all of the samples. In addition, for each reporter strain used, a standard curve was established applying synthetic AHL, thus it could be considered as a quantitative approach for AHL detection. In recent years, the work described before has been mentioned as an approach to identify the presence of AHL

in mixed culture wastewater treatment systems, since new methods are emerging in this scientific field.

2.3.3 Quorum sensing in granules

As described before, in the context of biological wastewater treatment, AGS has been recognized as a new approach for successful textile wastewater treatment including the biodegradation of azo dyes [2]. However, the mechanisms of granulation have not yet been fully understood and they seem to have an important role in the stability of the system.

Since biological granulation is one type of biofilm formation and the bacterial adhesion has been described as being highly dependent on AHL-regulatory systems, particularly from aspects relying on extracellular polysaccharides (EPS) formation [8], [9], QS analysis is extremely promising to elucidate the granulation mechanisms. Previous studies have documented the apparent impact of QS molecules in the beginning of the granulation process and also regarding granule stability, particularly, the biomass intracellular extract seemed to contain molecules responsible for granule formation [32].

One main issue is the extraction and fixation of the AHL extract from biomass samples. Ren et al. (2010) [32] reported a method to harvest AGS samples for AHL extraction. The aim of the work was to evaluate QS effect in the granulation process. Therefore, the method has been described in other experiments since then. An optimized system of a sectional membrane bioreactor was used in order to evaluate the influence of QS molecules in granulation. The results suggested that the AHL production could have impact on initial cell attachment and in granule formation. Furthermore, the impact of intracellular extract from mature granules was evaluated and it was demonstrated their importance in accelerating the sludge granulation process.

In the last couple of years two papers were released focusing on AGS systems, which analyzed the AHL interaction with the granules' properties. In Lv et al. (2014) [9], it was investigated the attachment potential of AGS and conventional flocculent activated sludge (FAS). Besides the finding that AGS had higher AHL content than FAS, the work also refers a denser structure in EPS. In addition, specific bacterial communities have been described exclusively in AGS. From another experiment from 2014, Li et al. [8], investigated AHL-based QS in AGS cultivated in SBR. However, the methodology followed was only qualitative, and did not allow for quantitative measurement of the concentration of AHL in the samples.

In the present work the approach followed to measure AHL content was similar to the described in Li et al. (2014) and Lv et al. (2014) [8], [9] however, the method of [8] was adapted to determine the AHL concentration in a quantitative way.

3 Materials and Methods

3.1 Operations

3.1.1 Feed

The carbon source used was a starch-based sizing agent commonly employed in the textile industry, Emsize E1 (Emsland-Stärke GmbH, Germany) and the stock solution was prepared as described in Lourenço et al. (2000) [6], diluting Emsize E1 in distilled water to a final concentration of 100 g/L.

The azo dye solution used was an acid red 14 solution (AR14, Chromotrope FB,Sigma-Adrich, 50% dye content) at 3.0 g/L of concentration.

A silver nanoparticles (AgNPs) suspension was used and it was prepared with a silver nanopowder (Silver nanopowder <100 nm particle size, Sigma-Aldrich) to a concentration of 50 mg/L.

The final feed solution, which represents a synthetic wastewater, was prepared adding the azo dye to an initial concentration of 40 mg/L and diluting the carbon source stock solution in distilled water to a chemical oxygen demand (COD) content of 1000 mgO_2/L (1.15 g/L Emsize E1) and adding buffering phosphates and nutrients to the following concentrations: 2310 mg/L $Na_2HPO_4.12H_2O$, 762 mg/L KH_2PO_4, 143 mg/L NH_4Cl, 22.5 mg/L $MgSO_4.7H_2O$, 27.5 mg/L $CaCl_2$, 250 µg/L $FeCl_3.6H_2O$, 40 µg/L $MnSO_4.4H_2O$, 57 µg/L H_3BO_3, 43 µg/L $ZnSO_4.7H_2O$, 35 µg/L $(NH_4)_6Mo_7.4H_2O$. The COD mass ratio was 100:3.7:3.7. In order to avoid the nitrification process it was used low N supply.

For shock tests, azo dye and Emsize E1 concentrations were increased to 60 mg/L and 3000 mgO_2/L, respectively in the feed solution at the end of operation 2 (days 91 and 98, respectively).

Shock tests were also performed during operation 4 increasing the azo dye concentration to 120 mg/L at day 160 and the Emsize E1 concentration to 3000 mgO_2/L at day 167 of operation. Then, the feed returned to the initial concentrations at day 175. In addition, instead of adding 1000 mgO2/L of Emsize E1 at day 288 of operation it was added 500 mgO2/L of Emsize E1 and the same amount of acetate. At the end of operation, the carbon source was changed and it was added 1000 mgO2/L of acetate.

3.1.2 Sequencing batch reactors (SBRs) systems

Five different operational periods were considered. The SBRs addressed in all the studies had 1.5 L of working volume and a height/diameter ratio of 2.5.

The synthetic wastewater was fed with an exchange ratio of 50%, an organic loading rate of 2.0 $kgCODm^{-3}d^{-1}$ and an initial azo dye concentration of 40 mg/L except for the dye-free control SBR used in operation 2. In operation 5 it was added also 10 mg/L of AgNPs in SBR1.

The reactors were inoculated with different biomass samples according to the purpose of the study and were operated at room temperature without pH control. In operations 2, 3 and 4 the SBRs were inoculated with aerobic granular sludge (AGS). However, in the first and fifth operations the SBRs were inoculated with conventional activated sludge (CAS).

The 6 h cycle in each operation included a fill step of the synthetic textile wastewater, a reaction time with a mixing anaerobic stage where the mixing was provided by magnetic stirrers (except for SBR2 in operation 3) followed by aeration which was supplied by air compressors at the bottom of the reactors (2 vvm) and a drain phase. The different operational conditions of the five operations tested are summarized in **Table 3.1**.

Table 3.1: General description of the five operational conditions tested. Operational period, inoculum source, features of the hydrodynamic regime and cycle phases in each reactor.

	Time (days)	Inoculum	Features	Cycle description
1	33	Conventional activated sludge from Chelas WWTP	**SBR1**: dye-fed with intermittent aeration	**SBR1**: 30 min anaerobic mixing and 20 min aeration
2	102	Aerobic granular sludge from Frielas WWTP	**SBR1**: dye-fed, fast static fill **SBR2**: dye-free, fast static fill	**SBR1** and **SBR2**: 1.5 h anaerobic mixing and 3.5 h aeration
3	139	Aerobic granular sludge from operation 2 stored during 6.5 months at 4 ºC	**SBR1**: dye-fed, fast static fill **SBR2**: dye-fed, anaerobic plug-flow fill	**SBR1**: 1.5 h anaerobic mixing and 3.5 h aeration **SBR2**: 2 h anaerobic plug-flow fill, 3.5 h aeration
4	315	Aerobic granular sludge from operation 3 stored during 3 months at 4 ºC	**SBR1**: dye-fed, fast static fill **SBR2**: dye-fed, anaerobic plug-flow fill	**SBR1**: 1.5 h anaerobic mixing and 3.5 h aeration **SBR2**: Until day 179 - 50 min anaerobic plug-flow fill, 1.5 h anaerobic mixing and 3.5 h aeration From day 180 - 1.33 h anaerobic plug-flow fill, 1 h anaerobic mixing and 3.5 aeration
5	281	Conventional activated sludge from Chelas WWTP	**SBR1**: dye- and AgNPs-fed, fast static fill **SBR2**: dye-fed, AgNPs-free, fast static fill **SBR3**: dye-fed, AgNPs-free, anaerobic plug-flow fill	**SBR1** and **SBR2**: 1.5 h anaerobic mixing and 3.5 h aeration **SBR3**: 1.5 h anaerobic plug-flow fill, 30 min anaerobic mixing and 3.5 h aeration

In operation 1, the granulation occurred during the operation using CAS as inoculum, the reactor was operated in a fast static fill followed by mechanically mixed anaerobic phase (30 min) and aeration (20 min).

Two reactors were run in parallel in operation 2 and both were inoculated with AGS. The SBR1 was fed with the synthetic textile wastewater with dye and the SBR2 was used as a control without dye in the feed solution. Both of the reactors were operated in a fast static fill followed by mechanically mixed anaerobic phase (1.5 h) and aeration (3.5 h).

It was in the operation 3 that it was added another hydrodynamic regime and the inoculum used was the AGS stored from the operation 2 during 6.5 months at 4 ºC. This operation consisted in 2 different reactor systems. SBR1 was operated in a fast static fill followed by mechanically mixed anaerobic phase (1.5 h) and aeration (3.5 h). The other system (SBR2) included an anaerobic plug-flow fill phase (2 h) from the bottom of the reactor followed by an aerobic phase (3.5 h).

For operation 4 the two hydrodynamic regimes were maintained and the biomass from the previous operation it was used as inoculum. SBR1 was operated in a fast static fill followed by mechanically mixed anaerobic phase (1.5 h) and aeration (3.5 h) and SBR2 was operated in an anaerobic plug-flow fill phase of 50 min, 1.5 h of anaerobic mixing and 3.5 h of aeration until day 179. At day 180 it was imposed 1.33 h of anaerobic plug-flow fill followed by 1 h of anaerobic mixing and 3.5 h of aeration.

In operation 5 the granulation occurred during the operation (as occurred in the first operation). SBR1 and SBR2 were operated in a fast static fill followed by mechanically mixed anaerobic phase (1.5 h) and aeration (3.5 h) and SBR3 was operated in an anaerobic plug-flow fill phase of 1.5 h, 30 h of anaerobic mixing and 3.5 h of aeration. It was added 10 mg/L of AgNPs only in SBR1.

The granulation occurred during the run, by imposing a gradual reduction in the settling time. At first, for the operation 1 it was imposed a settling time of 40 min and after 6 days it was reduced to 10 min and at day 8 it was again reduced to 6 min until the end of the operation. The granulation of the fifth operation occurred decreasing the settling time during three weeks as described in **Table 3.2**.

Table 3.2: Settling times imposed for granulation process during the fifth operation.

Operation day	1	3	7	10	14	17	24	28	28 - 281
Settling time (min)	60	40	30	20	15	10	7	5	5

Samples were periodically collected in order to monitoring the SBRs performance. Chemical oxygen demand (COD) analyses were performed as well as UV-Vis in order to followed the percentage of COD and color removal, respectively (**Table 3.3**). It was also assessed the total

suspended solids (TSS) analyses of the effluent (**Table 3.4**), and the sludge volume index at 5 min (SVI_5) and at 30 min (SVI_{30}) was measured to follow the settling properties of the granules. All these analyses were performed previously to the work developed in this master thesis. However, some data are described in order to discuss the results obtained regarding mixed microbial culture characterization and quorum sensing analyses.

Table 3.3: Relevant data of percentages of chemical oxygen demand (COD) and color removal detected during the operations.

Operation	SBR	Operation day	Color removal (%)	COD removal (%)
1	**SBR1**	28	80	80
2	**SBR1**	37	80	
		58	60	
		79	80	
		100	80	
3	**SBR1**	70	80	
	SBR2	70	30	
4	**SBR1**	288	80	
		307	15	
5	**SBR1**	36	80	
	SBR2	8	80	
		22	45	
		36	80	
	SBR3	36	60	

Table 3.4: Total suspended solids (TSS) in g/L detected in the effluent of the SBR1, SBR2 and SBR3 of the operation 5 during the days of the granulation process.

	TSS (g/L)		
Operation day	**SBR1**	**SBR2**	**SBR3**
1	2.98	1.65	4.67
7	1.93	1.06	1.06
14	0.448	0.103	0.175
21	0.098	0.185	0.093
28	0.179	0.948	0.948
31	0.103	0.125	0.080
35	1.09	1.30	0.368
38	0.186	0.160	0.073
43	1.10	0.565	0.204
52	0.345	0.145	0.228

3.2 Fluorescence *in situ* hybridization (FISH) analysis

3.2.1 Sample fixation

The biomass samples (0.5 mL) were harvested from the reactors at the same height and were fixed with 4% paraformaldehyde – PFA (1:3 proportion) [35]. It was added 1.5 mL of PFA 4% and the sample was stored at 4 ºC from 1 to 3 h. After this period the biomass was centrifuged at 6 000 rpm for 3 min and then the pellet was washed (two times) with 1 mL of PBS 1x and again centrifuged at the same specifications. The cells were re-suspended with 0.5 mL PBS 1x and 0.5 absolute ethanol at -20 ºC and finally, agitated with a vortex and stored at -20 ºC.

Biomass samples were selected to proceed with the characterization according to significant developments detected over each operation.

3.2.2 Probes

The fluorescently labelled oligonucleotide probes used for FISH experiments were: Fluorescein isothicyanate (FITC)-labelled EUBmix probe for identification of all Bacteria (mixture of EUB338 [36], EUB338-II and EUB338-III [37]) and Cyanine 3 (Cy3)-labelled ALF969 for *Alphaproteobacteria* [38]; BET42a for *Betaproteobacteria* and GAM42a for *Gammaproteobacteria* [39]; CF319a for most *Flavobacteria,* some *Bacteroidetes* and some *Sphingobacteria* [40]; HGC69a for *Actinobacteria* [41]; LGCmix (mixture of LGC354A, LGC354B and LGC354C – [42]) for *Firmicutes*; SuperDFmix (mixture of TFOmix – TFO_DF218 and TFO_DF618 [43], DFmix – DF988 and DF1020 [44] and DF198 [45]) for *Defluvicoccus vanus* related glycogen-accumulating organisms (GAOs), and Par651 for *Paracoccus* [46].

3.2.3 Slide preparation

According to Amann et al. (1995) [36] the FISH slides were carried out as following. 5 uL of homogenized biomass samples were applied in each well. Then the slides were dried with compress air. After that were dehydrated in ethanol series (3 min in each): 50%, 80% and 98% ethanol and once more dried with compressed air.

For the probe hybridization, a tissue paper was folded and cut to fit in a Falcon tube and a hybridization buffer was prepared in a 2 mL Eppendorf (360 µL NaCl 5 M, 40 µL TRIS-HCl 1 M, MilliQ Water, 2 µL SDS 10% and formamide according to the percentage required for each probe [see **Table 3.5** for the amount]). 8 µL of the hybridization buffer were applied in each well in the fume hood and the remainder solution was used to moisture the tissue in the Falcon tube. Then 0.5 µL of the probes were applied in each well and gently mixed with the buffer. Finally, the slide was put into the falcon tube and placed in the oven at 46 ºC for 2 h.

Table 3.5: Amount of formamine and MilliQ water to prepare the hybridization buffer.

% Formamide	Probes	MilliQ water amount (µL)	Formamide amount (µL)
25	HGC69a	1098	500
35	Alf969 Bet42a Gam42a CF319a	898	700
40	Par651	798	800

After the incubation, a washing buffer was prepared in a 50 mL Falcon tube (NaCl 5 M [see **Table 3.6** for the amount], 1 mL TRIS-HCl 1 M, EDTA 0.5 M [see **Table 3.6** for the amount], water up to 50 mL and 50 µL SDS 10%) and placed in a bath until reaching 48 ºC, then the slide was washed in the fume hood with some drops of the buffer and immersed in the Falcon tube followed by an incubation in the bath for 10 min at 48 ºC. The slides were taken out from the bath, washed with MilliQ water at 4 ºC and dried with compressed air in order to quick remove the droplets.

Table 3.6: Amount of NaCl and EDTA to prepare washing buffer.

% Formamide	Probes	NaCl amount (µL)	EDTA amount (µL)
25	HGC69a	1490	500
35	Alf969 Bet42a Gam42a CF319a	700	500
40	Par651	460	500

Finally, the slides were covered using the anti-bleaching Vectashield and a cover slip placed gently and, after cleaning the excess with a tissue paper, nail polish was applied to the board of the cover slip. The slides were stored at -20 ºC.

3.2.4 Image acquisition and abundance assessment

Biomass samples were observed using an Olympus BX51 epifluorescence microscope (Japan) at 1000x, equipped with a camera.

Flourescence isothycionate (FITC) and the iodocarboamide dye Cy3 were excited with an argon laser (488 nm) and a helium neon laser (543 nm) and their emissions collected with 500 to 530 nm BP and 550 to 625 nm BP, respectively. Each sample and set of probes were carefully examined and a semi-quantitative assessment of the relative abundance of each specific probe was determined by comparison with the number of cells targeted by EUBmix, which corresponds to the total bacteria.

3.3 Quorum sensing analysis

3.3.1 Strains and growth conditions

Agrobacterium tumefaciens strain NTL4 and *A. tumefaciens* NTL4 (pZLR4) were kindly provided by Ramón Peñalver from Instituto Valenciano de investigaciones Agrarias, Valencia, Spain. Both strains lack the Ti plasmid thus, could not produce detectable AHL levels. Plasmid pZLR4 contains a vector conferring resistance to gentamicin and carbenicillin and other genes include a *traG::lacZ* fusion and *traR* which confers the ability to produce a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) and, hence, an indicator of AHL presence. *A. tumefaciens* NTL4 was used as negative control.

Whereas *A. tumefaciens* NTL4 (pZLR4) grows in minimal medium or LB broth supplemented with 50 µg/mL of gentamicin and incubated overnight at 28 ºC with shaking at 150 rpm, *A. tumefaciens* NTL4 grows in minimal medium and LB without any additional antibiotic.

First, both strains were grown in a pre-inoculum of 5 mL of liquid LB medium (NTL4 (pZLR4) with 5 µL of gentamicin (50 µg/mL)) at 28°C, at 150 rpm in the orbital shaker, and transferred to a 100 mL Erlenmeyer with 30 mL of LB medium, (NTL4 (pZLR4) with the same antibiotic concentration) and both at the same conditions, until OD_{600} > 1 in order to prepare cryovials with 20% of glycerol and stored at -80ºC to generate cell banks. The cultures were followed during 24 h in order to obtain a growth curve (Appendix I).

3.3.2 Preliminary qualitative AHL detection

This analysis was performed in order to verify the presence of AHL in the mixed microbial culture wastewater treatment system. The experiment was only applied to SBR2 of operation 5.

The procedure used for AHL detection was based on the method described in Ravn et al. (2001) [30]. An assay was performed by diluting an overnight-grown fresh culture of *A. tumefaciens* NTL4 (pZLR4) in LB with 50 µg/mL of gentamicin at 28 ºC and 150 rpm, in melted LB agar in 1:2 ratio and then, dispensing it in culture dishes as quickly as possible. After solidification, 10 µL of X-Gal (20 mg/mL in dimethyl sulfoxide - DMSO) were spread over the surface. A control assay was made using *A. tumefaciens* NTL4 instead of the reporter strain NTL4 (pZLR4)

The equivalent volume to 10 mg of dry biomass was harvested from one reactor (control reactor). Then, the biomass was washed as followed: after biomass settling, 500 µL of the supernatant were removed. Then, the biomass was washed 3 times with 1 mL of PBS 1x. The gently washed fresh biomass samples from SBR2 of the operation 5 were placed on the plate center. And finally, the assays were incubated overnight at 28 ºC.

3.3.3 AHL quantification

3.3.3.1 AHL extraction

The QS analyses were performed only during operation 5 (**Table 3.1**). Mixed liquor samples with an equivalent amount of 10 mg of dry biomass were harvest from the three bioreactors at the end of one cycle twice a week, during the granulation phase. Then, the AHL extraction procedure was performed based on Ren et al. (2010) [32] method. First, the sludge sample was centrifuged at 6 000 rpm for 10 min at 4 ºC in order to remove the supernatant S1. The supernatant S1 was stored at -20 ºC and the retained pellet was re-suspended in 1.5 mL MilliQ water, in order to proceed to the next step. Second, the suspended sludge mixture was sonicated for 30 min at 30 W/mL in an ice bath to break up bacterial cells. After that, the suspension was centrifuged at 10 000 rpm for 10 min and 4 ºC, and finally the supernatant S2 was collected and stored at -20 ºC.

3.3.3.2 AHL measurement

The method for the measurement of AHL content was based on the method described in Singh & Greenstein (2006) [33]. The reporter strain *A. tumefaciens* NTL4 (pZLR4) was incubated to late exponential phase (16 h to 18 h) in a rotary shaker at 28 ºC and 150 rpm in minimal medium (2 g/L glucose, 10.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 15 mg/L $CaCl_2 \cdot 2H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$ and 3 mg/L $MnSO_4 \cdot H_2O$) supplemented with 50 µg/mL of gentamicin. The bacterial culture was diluted in fresh minimal medium lacking gentamicin to an OD_{600} of 0.1 and 1 mL dispensed in sterilized tubes, which contained 1 mL of supernatant S2 from the reactors. A control sample was performed at this point in the same conditions, using the reporter strain culture incubated with MilliQ water instead of supernatant S2. Then the tubes were incubated again in a rotary shaker at 28 ºC, with 150 rpm for 16 h to 18 h. After that, 200 µL from each incubated mixture were dispensed into an Eppendorf and 50 µL of X-Gal (20 mg/mL in DMSO) were also added. Then, each Eppendorf was agitated in vortex in order to homogenize the culture and was incubated for 2 h in darkness, at room temperature and 150 rpm of agitation.

The absorbance was measured at 615 nm using a microplate reader (Varian, Cary 50 Bio UV-Visible Spectophotometer). For this purpose, the suspensions were settled for 20 minutes and 100 µL of each Eppendorf were dispensed per well of 96-well plates (Greiner, Germany).

3.3.3.3 Calibration curve

Increasing amounts of a synthetic AHL (N-(3-oxo-dodecanoyl)-L-homoserine lactone - ODHL from Sigma-Aldrich) were inoculated with the reporter strain in order to achieve a relationship between ODHL concentration and OD_{615}. The protocol followed was similar to the one described before.

The reporter strain *A. tumefaciens* NTL4 (pZLR4) was incubated to late exponential phase (16 h to 18 h) in a rotary shaker at 28 ºC and 150 rpm in minimal medium supplemented with 50 µg/mL of gentamicin. The bacterial culture was diluted in fresh minimal medium without gentamicin to an OD_{600} of 0.1 and 1 mL dispensed in sterilized tubes which contained 1 mL of ODHL solution (initial concentrations between 0 nM and 37.5 nM). Then the tubes were incubated in a rotary shaker at 28 ºC, with 150 rpm for 16 h to 18 h. After that, 200 µL from each incubated mixture were dispensed into an Eppendorf and 50 µL of X-Gal (20 mg/mL in dimethyl sulfoxide) were also added. Then each Eppendorf was agitated in vortex in order to homogenize the culture and incubated for 2 h in darkness at room temperature and 150 rpm of agitation.

The absorbance was measured using a microplate reader (Varian, Cary 50 Bio UV-Visible Spectophotometer) at 615 nm. For this purpose, the suspensions were settled for 15 min and 100 µL of each Eppendorf were dispensed per well of 96-well plates (Greiner, Germany).

4 Results and Discussion

4.1 Characterization of mixed microbial community: Fluorescence *in situ* hybridization (FISH)

Using specific group probes, FISH analysis allowed the identification of microbial populations and also provided information regarding the relative abundance of different bacterial populations within the samples analyzed. The probes used in all the FISH experiments were selected to cover the core community typically detected in wastewater treatment plants (WWTP) as detailed above [23].

For all the samples the fluorescently labelled oligonucleotide probe, (FITC)-labelled EUBmix probe, was used in addition with one of the more specific probes previously described in the methods (Cy3)-labelled probes (Alf969, Bet42a, Gam42a, CF319a, HGC69a, LGCmix).

In this section the semi-quantitative FISH results concerning the broad study performed for each operation conditions are graphically presented, in conjunction with the results discussion, taking into account the relevant episodes occurred during the operation. The most relevant results are exemplified with FISH images.

4.1.1 First operation – Granulation in intermittent aeration regime

Biomass samples from the conventional activated sludge inoculum provided by Chelas WWTP in 20/03/2013 and a sample taken at the last day of operation (day 28) were analyzed in order to understand the effect of the operating conditions in the original microbial community.

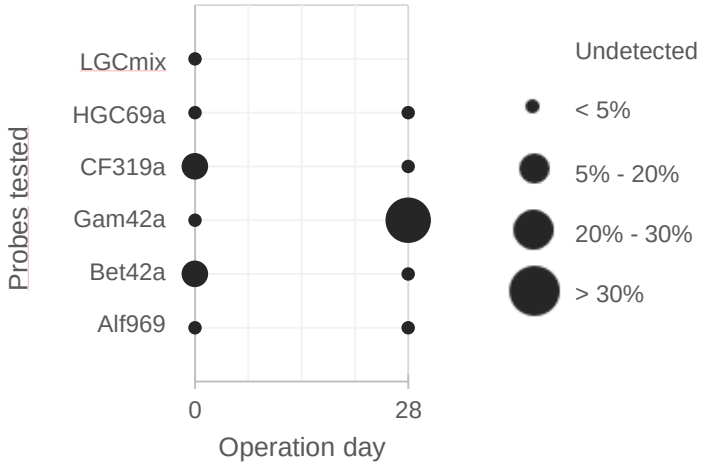


Figure 4.1: Abundance of the select *Bacteria* in the mixed microbial culture samples from the bioreactor fed with synthetic textile wastewater containing dye, operated in intermittent aeration during the first operation period, from days 0 and 28 detected by FISH analysis.

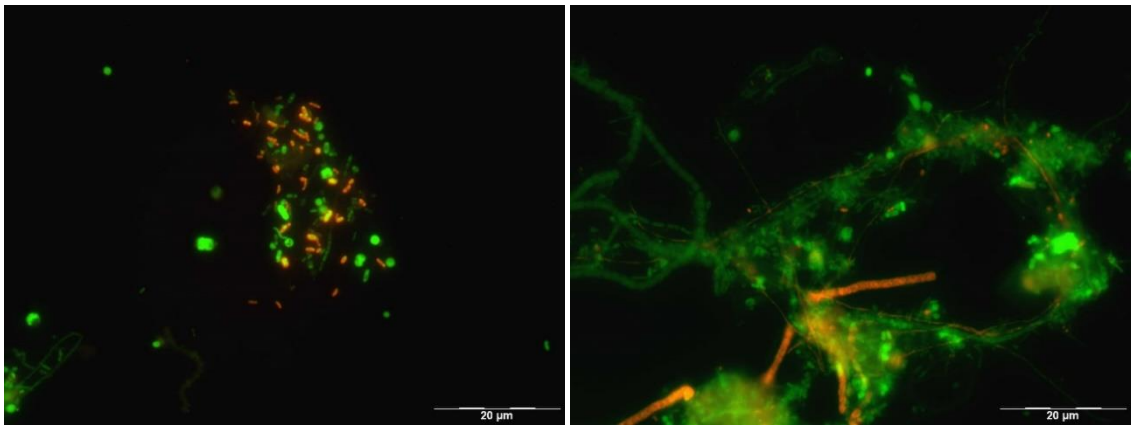


Figure 4.2: FISH images of the bioreactor inoculum of the operation 1, at 1000 x. The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probes Bet42a (left) and CF319a (right), both red. Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

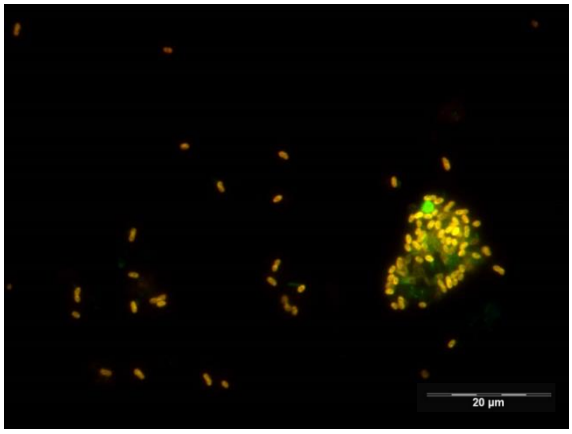


Figure 4.3: FISH image of the biomass sample at day 28 from the bioreactor operated in intermittent aeration of the operation 1, at 1000 x. The image shows bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Gam42a (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

FISH analyses allowed the identification of the major bacterial groups as belonging to the *Proteobacteria*. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were identified in both the samples analyzed (**Figure 4.1**). However, it was observed low abundance of *Alphaproteobacteria* and *Gammaproteobacteria* in the inoculum while *Betaproteobacteria* populations were apparently the most abundant groups detected. In addition, it was identified a significant presence of filamentous *Cytophaga-Flavobacteria* populations and it was also confirmed the presence of High and Low G-C content Gram-positive bacteria in low abundance in the original biomass, by the positive signal given by (Cy3)-labelled probes CF319a, HGC69a and LGCmix, respectively.

As expected, the inoculum had a large variety of microbial populations however, it must be mentioned that, a significant part of the biomass sample (at least 50%) was not covered by the (Cy3)-labelled probes selected, indicating a diverse initial community, containing many smaller groups of organisms other than those typically found in a WWTP core community. However, (FITC)-labelled EUBmix revealed positive signal for nearly all the cells in the sample which indicates the prevalence of bacteria as compared to other microbial domains.

The microbial community within the reactor totally changed from the first day to the last day of the study period, after 28 days subjected to specific reactor operation settings. Conditions that likely most influenced the microbial populations' selection in this period include the intermittent aeration regime imposed, the presence of the dye and other textile effluent components (sizing agent, phosphorus etc) and also the sedimentation time used. The abundance of *Cytophaga-Flavobacteria* and *Betaproteobacteira* had a slight decrease from the first day to the last one and the signal of the (Cy3)-labelled probe LGCmix was not detected in the sample taken from day 28. Furthermore, the large dominance of *Gammaproteobacteria* on this date suggested that the color removal could be performed by a microbial community within the *Gammaproteobacteria* group.

In the present operation were also detected granules with poor settling characteristics. Furthermore, the unevenness detected regarding the mixed microbial culture might have indicated that the enrichment in *Gammaproteobacteria* cannot promote the granules stability and it might be required a more diverse culture.

4.1.2 Second operation – Impact of variable SRT and shock loads

FISH analyses were carried out with the aerobic granular sludge inoculum provided by Frielas WWTP in 23/04/2013 and with samples taken at days 37, 58, 79 and 100 of operation. They were analyzed in order to observe the effect of the operating conditions changes in the microbial community. It was imposed a sludge retention time (SRT) of 15 days from 42nd day of operation until 60th day and then shock tests were performed increasing the dye concentration and carbon source (Emsize E1) concentration three times higher than in the begging to 120 mg/L and to 3000 mgO_2/L, respectively.

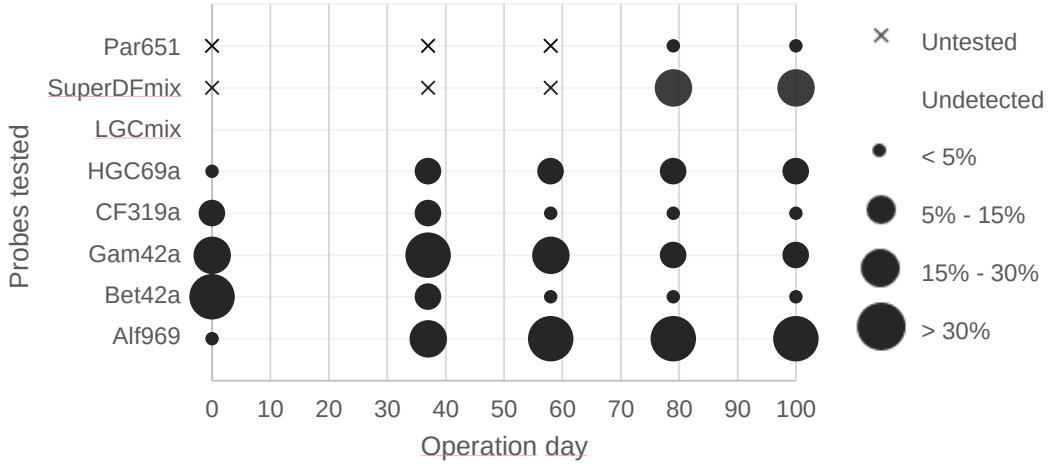


Figure 4.4: Abundance of the selected *Bacteria* in the mixed microbial culture samples from a bioreactor fed with synthetic textile wastewater containing dye, operated in fast static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) of the second operation, from day 0 to 100 detected by FISH analysis.

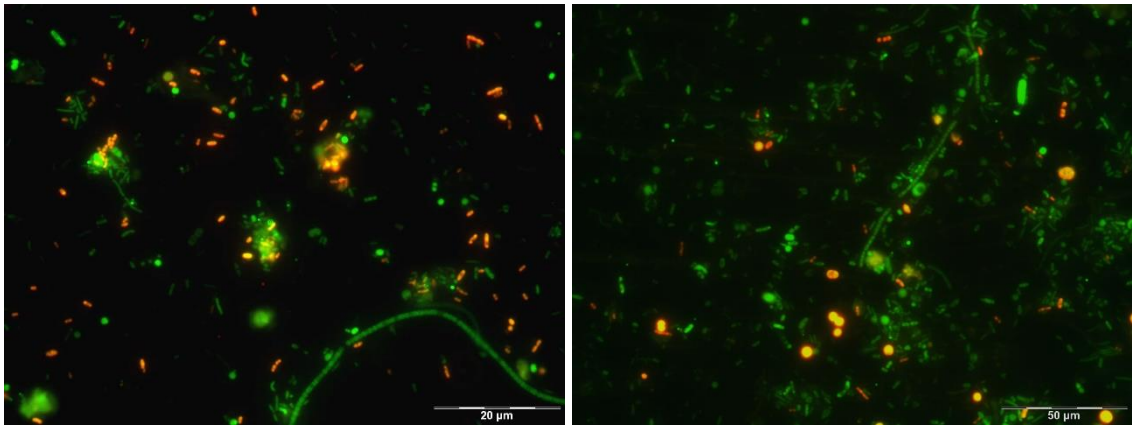


Figure 4.5: FISH images of the bioreactor inoculum of operation 2 at 1000 x. The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probes Bet42a (left) and Gam42a (right), both red. Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

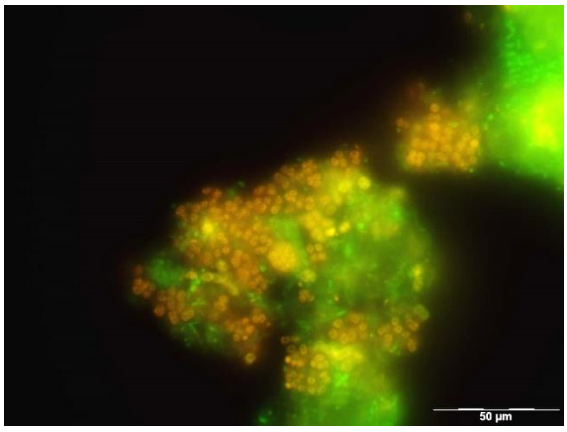


Figure 4.6: FISH image of the biomass sample at day 58 from the dye-fed bioreactor operated in fast static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h), of the operation 2 at 1000 x. The image shows bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Alf969 (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

The inoculum used in this second operation had a distinct mixed microbial community pattern when compared with the flocculent inoculum used in the previous operation. Therefore, the comparison between operations must be done carefully. As mentioned before, in the present operation the reactor was operated in 6 h cycles with 1.5 h of anaerobic mixing and 3.5 h of aerobic phase thus, the relation between this operation and the first one could give new insights regarding the effect of the operating conditions in the microbial community.

The inoculum contained a high abundance of *Betaproteobacteria,* overall with the same rod bacterial shape detected in the inoculum of operation 1. As occurred before, the *Betaproteobacteria* populations seem to be outcompeted during the operation period, as opposed to the *Alphaproteobacteria* group which apparently have organisms that have grown in the imposed conditions.

From the inoculum day to day 37 it was observed an evolution of the microbial community. The SRT of 35 days, imposed until day 37, allowed the growth of *Gammaproteobacteria*

populations. Furthermore, during this period, 80% of the color was removed in a SBR cycle, again indicating the possible association of a bacterial population from the *Gammaproteobacteria* group to the decolorization process.

From day 42 to 60 a SRT of 15 days was tested which had impact not only on the percentage of color removal (as descried in **Table 3.3**, decreased to 60%) but also, on the microbial community itself. After this phase, the sludge age was not controlled as in the beginning of the operation in order to recover the percentage of color removal achieved until day 46. Thus, 80% of color removal was successfully reached at day 79 (**Table 3.3**), however significant changes in the microbial populations were not detected, which showed the functional redundancy within the community for this purpose.

In addition, it was detected amine degradation since day 72 in the aerobic phase of the cycle. Regarding the importance of this finding the microbial community was evaluated in more detail, however it was just a slight change in the abundance of *Gammaproteobacteria* and also a change in *Alphaproteobacteria* communities in which the rod bacterial shape community was replaced by a cocci form bacteria growing in tetrads since day 58 (**Figure 4.6**). In view of these morphological findings, another two probes were used, (Cy3)-labelled probe SuperDFmix and Par651 in order to identify the *Alphaproteobacteria* communities that appeared in abundance since the 79th day of operation. As it is shown in **Figure 4.4**, the FISH experiments carried out with the two specific probes for *Alphaproteobacteria* communities allowed the identification of *Defluvicoccus vanus* related GAOs during this period, concomitant to a period where the detectable amine resulting from the azo bond cleavage was degraded.

The hybridization analyses allowed the assumption that amine degradation could be achievable by a specific community within one of the proteobacterial groups studied, since using the probes tested it was no significant evidence of microbial community changes regarding abundance from day 58 to 72. Furthermore, it could also be associated with an enzyme production by an existent group in some conditions. In this regard, *D. vanus* related GAOs can play an important role in mineralizing aromatic amines, since this group represents the majority of the *Alphaproteobacteria* dominant group.

Even more, shock tests were performed at the end of the operation (days 91 and 98). The concentration of dye and carbon source was increased in the feed. However, this changing did not substantially affect the abundance of the bacterial groups tested and the mixed microbial community remained apparently the same during the shock loads of these two feed components.

4.1.3 Third operation – Impact of storage in mixed microbial community

Biomass samples from the inoculum of this testing period and from day 70 of operation were analyzed in order to observe the effect of different hydrodynamic regimes in the microbial community. The inoculum used in the present operation was the aerobic granular sludge from the previous operation after six and a half months of storage at 4 ºC.

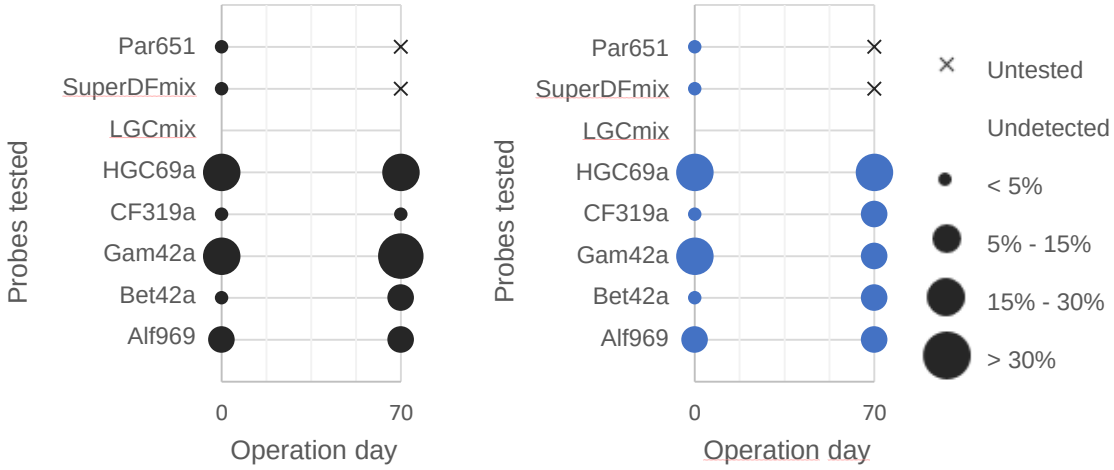


Figure 4.7: Abundance of the selected *Bacteria* in the mixed microbial culture samples from the dye-fed bioreactor operated in a static fill phase followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) (left) – SBR1, and from the dye-fed bioreactor operated in a plug-flow fill anaerobic phase (2 h) and an aerobic phase (3.5 h) (right) – SBR2 of the third operation, from day 0 to 70 detected by FISH analysis. The inoculum results are the same in both graphics.

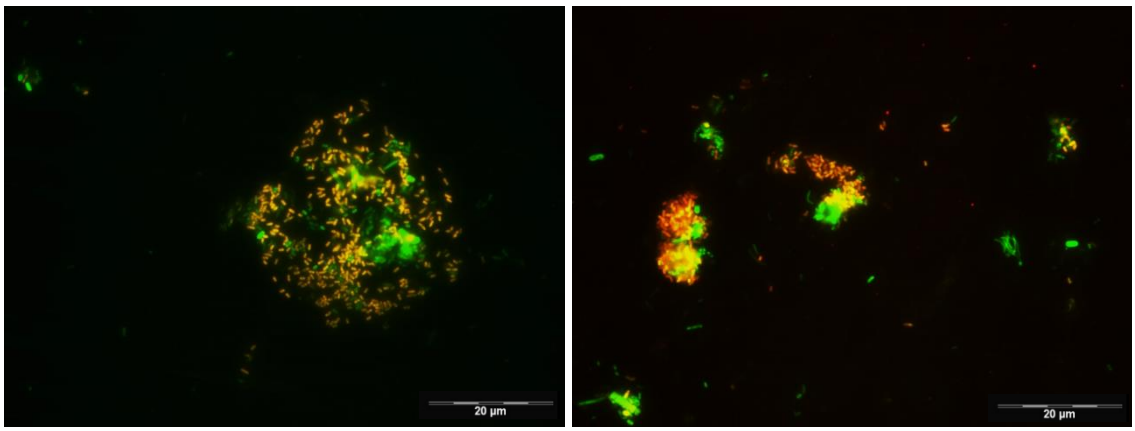


Figure 4.8: FISH images of the biomass inoculum used in both the dye-fed bioreactors of the operation 3 at 1000 x. The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probes Alf969 (left) and HGC69a (right), both red. Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

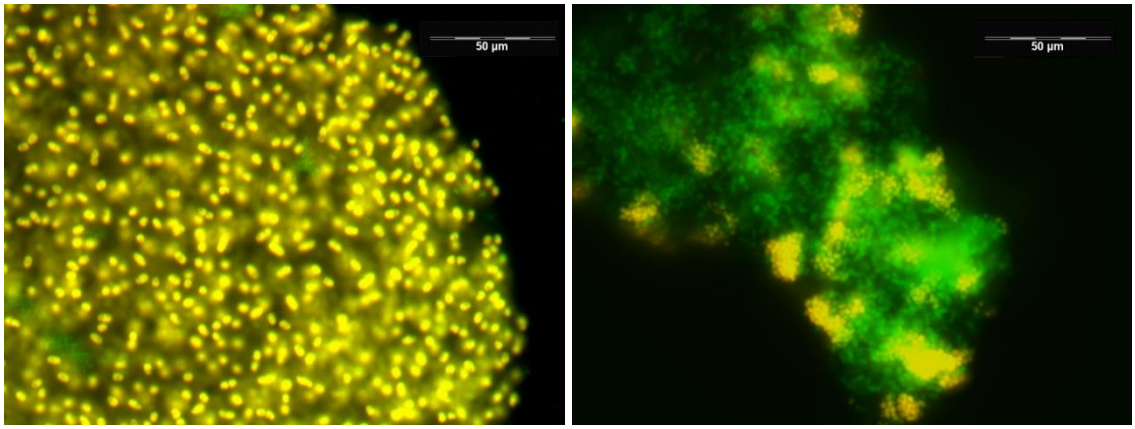


Figure 4.9: FISH images of the biomass sample at day 70 from the dye-fed bioreactors of the operation 3 at 1000 x. Bioreactor operated in static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) – SBR1 (left) and dye-fed bioreactor operated in a plug-flow fill anaerobic phase (2 h) and an aerobic phase (3.5 h) – SBR2 (right). The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Gam42a (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

The first issue to be addressed is the impact of the storage in the biomass. Regarding the proteobacterial (Cy3)-labelled probes, it was detected differences within the major bacterial groups. The *Alphaproteobacteria* populations were susceptible to storage since, the *Defluvicoccus vanus* related GAOs and genus *Paracoccus* were not so abundant in the sample after storage as in the biomass sample taken from the last day of the operation 2. This result is consistent with the hypothesis of the involvement of *D. vanus* related GAOs in the amine biodegradation since the mixed microbial community lost this capacity during the storage period. However, it was detected an *Alphaproteobacteria* population with a rod-shape bacteria morphology resistant to the storage (**Figure 4.8**). Moreover, in the proteobacterial context, *Gammaproteobacteria* represented an abundant group in the inoculum. The hybridization with HGC69a probe revealed also highly abundant communities of *Actinobacteria* after long storage, which could possibly be linked to the known capacity of some *Actinobacteria* of producing spores.

Despite the effect of the storage in the biomass, after inoculation it was evaluated the impact of the two hydrodynamic regimes in the bioreactors performance: a reactor operated in fast static fill phase (SBR1) and the other operated in a plug-flow fill phase (SBR2). Therefore, two parameters need to take into consideration, one is the percentage of color removal and the other is the percentage of chemical oxygen demand (COD) removal. The main difference was observed in the color removal (30% in SBR1 vs 80% in SBR2) which could be associated to the microbial community evolution during the operation (**Table 3.3**).

The plug-flow feeding imposed during 1.5 h in the SBR2 allowed for a spatially differentiated distribution of substrate concentration in the bioreactor. Therefore, the organisms in the bottom of the reactor have contact with higher feed concentrations than the upper organisms, at all times during the feeding phase. This created a wide variety of microniches that simultaneously fostered the growth of organisms with different substrate affinities, thus creating the conditions for an even growth of various groups. Indeed, the results revealed an even microbial community with very similar abundances of the 5 microbial groups assessed. On the

other hand, the completely mixed bioreactor was fed in 30 min followed by mixing. During this period, the microbial community is exposed to low feed concentrations in the reactor, since the new feed is mixed with the residual volume of the previous cycle. Therefore, completely mixed conditions tend to homogenize conditions inside the reactor, which promote the growth of those organisms that are better adapted to the imposed uniform conditions. The FISH results corroborate this hypothesis, where the static fill promoted the dominant growth of *Gammaproteobacteria* and *Actinobacteria*, followed by other groups represented with lower abundance in the community.

Furthermore, it is important to mention the abundance of a rod-shaped bacterial population that hybridized with Gam42a probe which represented the majority of the sample in the last day of operation of SBR1 and also the presence of a distinct morphological form observed at the time of the (Cy3)-labelled probe Gam42a hybridization, which had a cocci morphology identified in SBR2 (**Figure 4.9**). Therefore, the difference in color removal capacity observed for the two reactors could be related to this microbial culture variation (**Table 3.3**).

4.1.4 Fourth operation – Impact of feed solution changes in granules stability

The strategy followed in this operation was to analyze biomass samples from the inoculum and from days 152, 175, 250, 288 and 307 of both the reactors, in order to identify the populations in the mixed microbial culture able to resist to changes imposed in the feed composition.

In addition, the impact of the hydrodynamic regime in granules stability was also assessed. It is also important to mention that the inoculum was different from one reactor to another, since the biomass from the SBR1 and SBR2 of the previous operation was stored at 4 ºC for 3 months in separate and the reactors were inoculated with the analogous biomass from the previous operation.

The dye and Emsize E1 concentrations were increased to 120 mg/L and 3000 mgO_2/L respectively at day 160 and day 167. After that period, the feed solution returned to the initial concentrations of dye and carbon source, 40 mg/L and 1000 mgO_2/L, respectively. The addition of acetate as carbon source was tested at day 288 adding 500 mgO2/L of acetate and the same concentration of Emsize E1. Finally, at the end of operation it was added acetate in the feed as carbon source instead of Emsize E1.

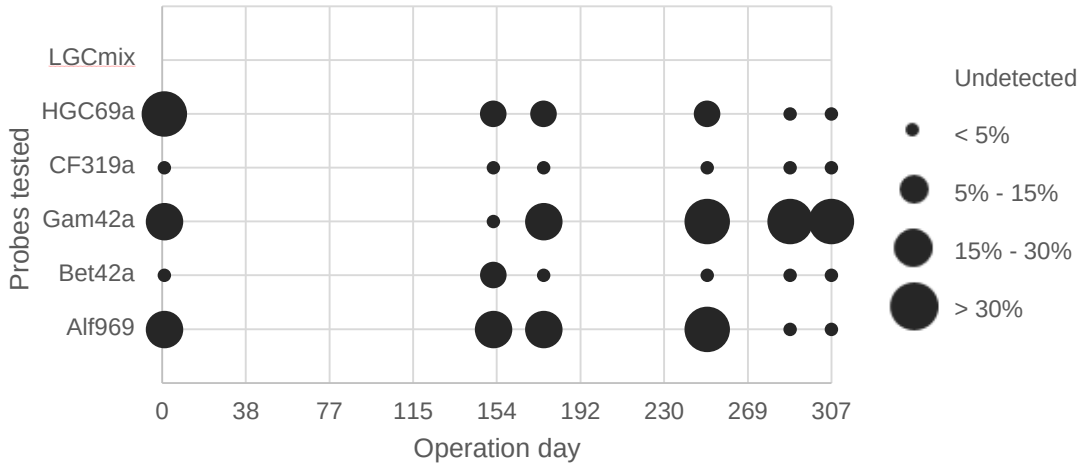


Figure 4.10: Abundance of the selected *Bacteria* detected by FISH analysis in the mixed microbial culture samples from the dye-fed SBR1 operated with a static fill phase, followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) during the fourth operation, from day 0 to 307.

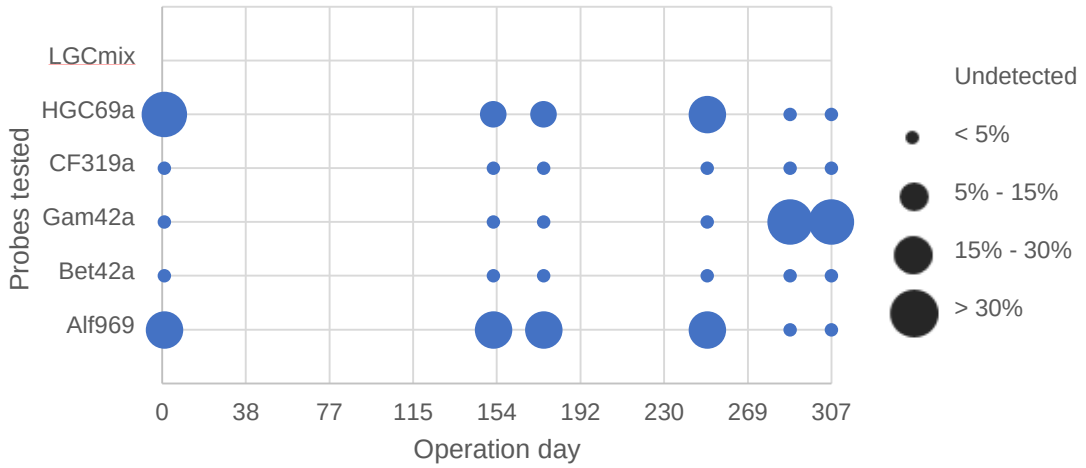


Figure 4.11: Abundance of the selected *Bacteria* detected by FISH analysis in the mixed culture samples from the dye-fed SBR2 operated with a plug-flow fill phase during the fourth operation, from day 0 to 307.

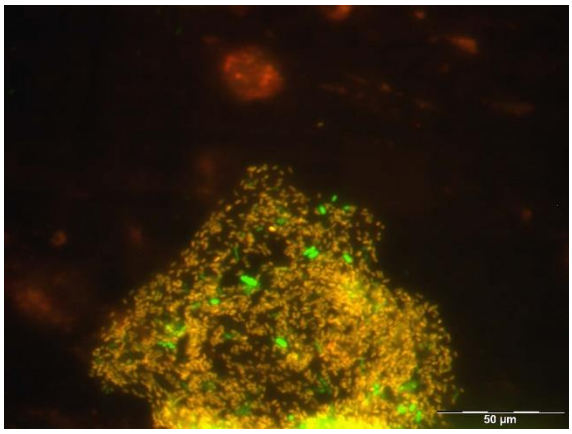


Figure 4.12: FISH image of the inoculum biomass sample from the dye-fed SBR1 operated with a static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) during operation 4 at 1000 x. The image shows bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Alf969 (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

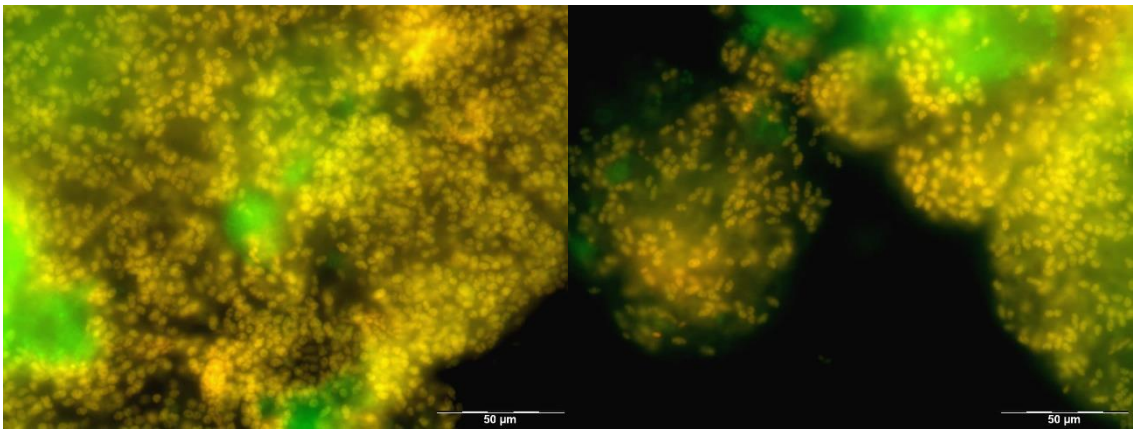


Figure 4.13: FISH images of the biomass sample at day 288 from the dye-fed bioreactors of the operation 4 at 1000 x. Bioreactor in operated with static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) – SBR1 (left) and plug-flow feed bioreactor – SBR2 (right). The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Gam42a (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

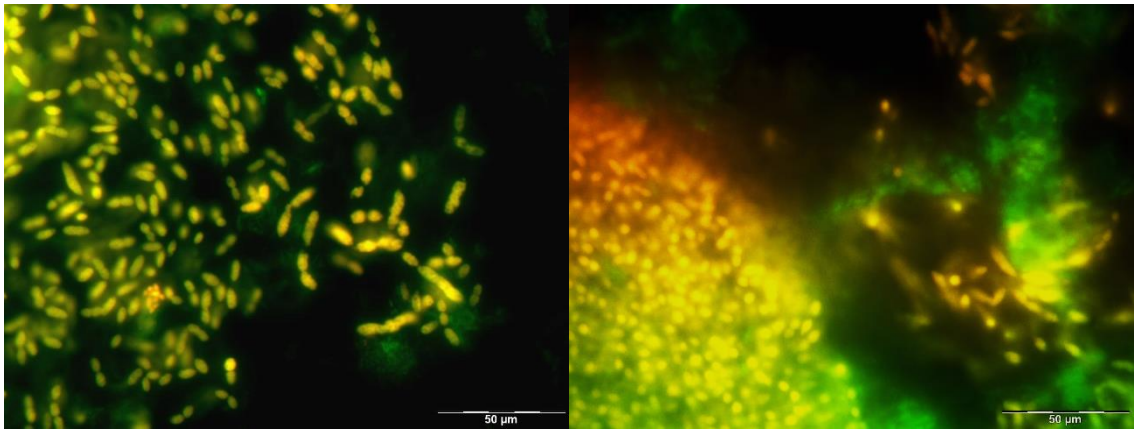


Figure 4.14: FISH images of the biomass sample at day 307 from the dye-fed bioreactors of the operation 4 at 1000 x. Bioreactor operated with static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) – SBR1 (left) and plug-flow feed bioreactor – SBR2 (right). The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Gam42a (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

As mentioned before, the SBR system in the fourth operation was the same that was established for the previous operation. Two SBRs fed with a synthetic textile wastewater were operated, one reactor operated in fast static fill phase (SBR1) and the other operated in a plug-flow fill phase (SBR2). However, it was introduced a mixing step in the anaerobic phase of the SBR2. Once more, the biomass was stored at 4 ºC and it was used to inoculate the reactors, however for a shorter time period of storage (3 months).

Regarding the reactor with a static fill, 1.5 h of anaerobic mixing and 3.5 h of aeration phase (SBR1), FISH analyses allowed the identification of three abundant groups that were resistant to a 3-month storage at 4 ºC. Namely, *Alphaproteobacteria* populations, *Gammaproteobacteria* and also High G-C content Gram-positive bacteria. The findings described in the previous operation that take into account the storage of biomass, were similar to the ones achieved in the present operation although, the abundance of *Alphaproteobacteria* major group were not so relevant in the inoculum of the third operation as in the present one. This fact could be associated to the presence of more resistant organisms within this proteobacterial class, in this regard it was detected a community with a rod-shape morphology, similar to the one detected in the third operation with the hybridization with (Cy3)-labelled probe Alf969 that seemed to outlast the storage (**Figure 4.12**).

At the end of third operation the bacterial communities identified within the *Gammaproteobacteria* major group were not morphologically similar (**Figure 4.9**). Which might be the cause for the abundance differences after storage regarding this group. Thus, it was detected less abundance of *Gammaproteobacteria* in the inoculum used for SBR2, the plug-flow feed bioreactor, than in the one used for SBR1. The highly hybridization of HGC69a and Alf969 was detected in both the inoculum samples.

One of the aims of the fourth operation was to evaluate if the introduction of a mixed anaerobic phase in SBR2, after the plug-flow fill, allowed the convergence of the mixed microbial

community. It was assessed the evolution of the microbial community in both the reactors and it was evaluated if the abundance of the microbial groups tested by the usage of FISH analysis was similar. For that the inoculum sample and the sample taken at day 152 from SBR1 and SBR2 were compared. Despite the presence of the major proteobacterial group *Betaproteobacteria*, the microbial community maintained similar relative abundances of each of the probe-targeted groups when comparing the inoculum with second sampling point (day 152), even regarding the differences in the inoculum sample.

The impact of dye and COD shock loads on the microbial community of the two SBRs was also assessed during the fourth operation, between days 152 and 175. For that, the dye amount was increased to 120 mg/L between days 160 and 174 and the COD was increased to 3000 mgO_2/L between days 167 and 174 in both reactors. However, the test had only impact in the microbial community of SBR1. The abundance of *Betaproteobacteria* decreased and the presence of *Gammaproteobacteria* organisms increased however, the presence of the dominant group *Alphaproteobacteria* remain unaltered after changing the dye concentration.

Finally, the impact of different carbon sources on the microbial community of the two SBRs was evaluated during the fourth operation, between days 250 and 314. However, the COD level of the feed solution was fixed at 1000 mgO_2/L

The replacement of 500 mgO_2/L of the feed COD from Emsize to acetate, from day 256 until day 291, allowed the selection of communities within *Gammaproteobacteria* major group on both the reactors and the color removal percentage remained the same (**Figure 4.13**).

However, when the feed COD corresponded to 1000 mgO_2/L of acetate, between days 292 and 314, the color removal of SBR1 decreased to 15% instead of the 80% removal registered before. This evidence was coupled with an evolution within the microbial community, not in terms of abundance regarding the hybridization of the probes used, but in the bacteria morphology. This might be an indication of the emergence of a new community within the *Gammaproteobacteria* group (**Figure 4.14**), more prevalent in the SBR1 than in SBR2, where the percentage of color removal was not affected.

It is important to notice that a granule collapse was detected in both reactors when was acetate introduced in the feed, which corresponded to the existence of an uneven culture that largely consisted in *Gammaproteobacteria* populations. This finding support the lack of granule stability reported in the 1st operation once the abundance of the major group *Gammaproteobacteria* in both cases was relevant.

4.1.5 Fifth operation – Impact of silver nanoparticles and of the SBR hydrodynamic regime in granulation

As for the first operation, in the present operation, the reactors were inoculated with conventional sludge provided by Chelas WWTP. Besides the usual two SBRs fed with a synthetic textile wastewater operated, one in fast static fill phase followed by 1.5 h of mixed anaerobic phase and 3.5 h of aerobic phase (SBR2) and the other operated in a plug-flow fill phase followed by 30 min of mixed anaerobic phase and 3.5 h of aerobic phase (SBR3), a third SBR (SBR1) was added to the system, and this one was fed with dye and silver nanoparticles (AgNPs) operated in a fast static fill phase followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h).

FISH analyses were carried out using a sample of the inoculum and samples collected at days, 36 and 106 for SBR1 and SBR3 and samples from days 8, 22, 36, 52 and 106 of operation to SBR2, since the purpose of the analysis was different for each reactor. The attempt was to assess the effect of the nanoparticles and once more, to evaluate the influence of the hydrodynamic regime in the granulation process.

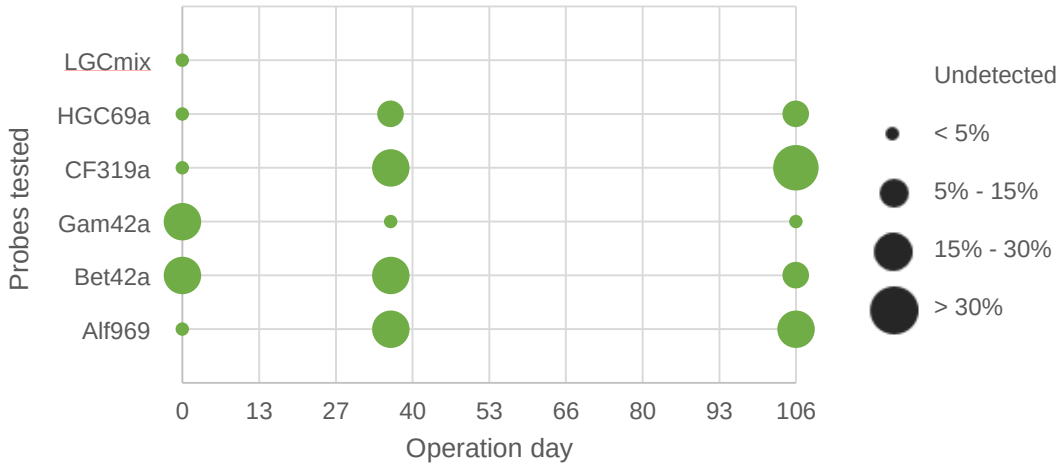


Figure 4.15: Abundance of the selected *Bacteria* detected by FISH analysis in the mixed microbial culture samples from SBR1 fed with an azo dye and AgNPs and operated with a static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) during the fifth operation, from day 0 to 106.

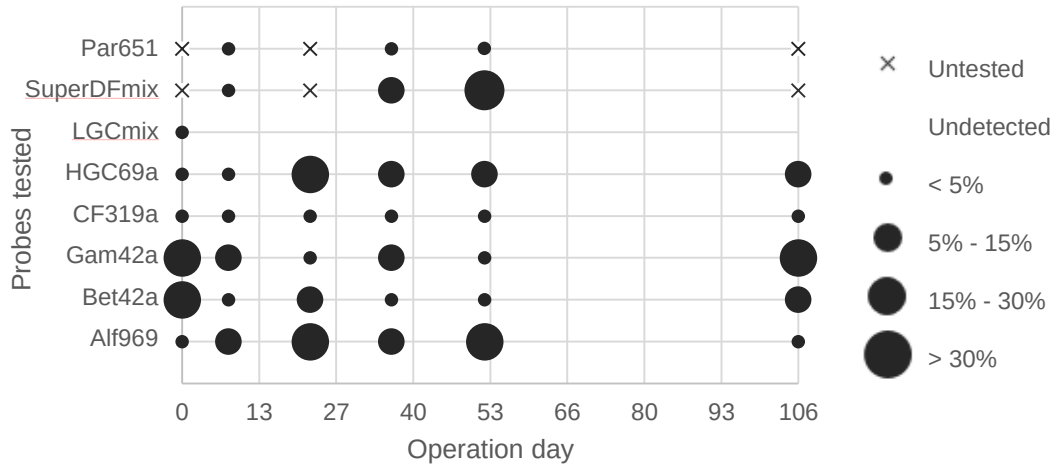


Figure 4.16: Abundance of the selected *Bacteria* detected by FISH analysis in the mixed microbial culture samples from the SBR2 fed with an azo dye and operated with a static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) during the fifth operation, from day 0 to 106.

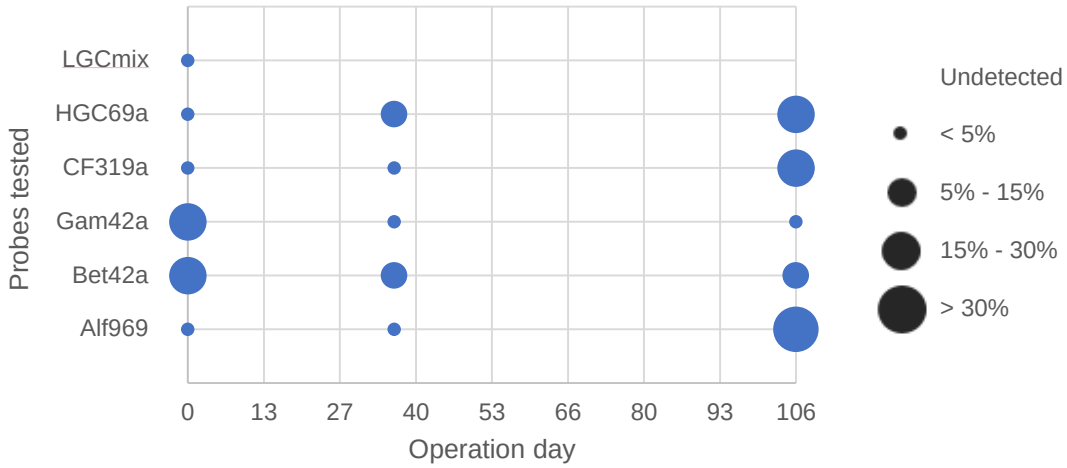


Figure 4.17: Abundance of the selected *Bacteria* detected by FISH analysis in the mixed microbial culture samples from the SBR3 fed with an azo dye and operated with a plug-flow feed phase followed by a mixed anaerobic phase (30 min) and an aerobic phase (1.5 h) during the fifth operation, from day 0 to 106.

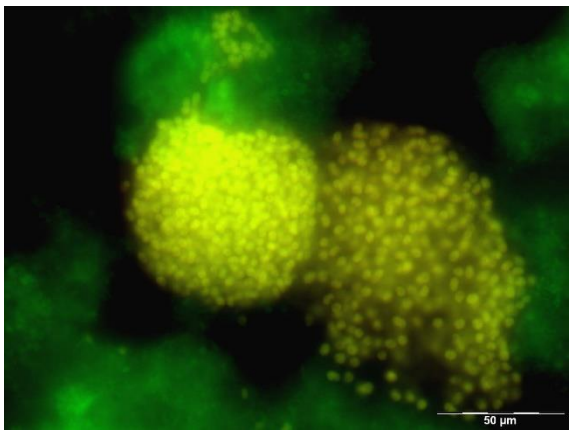


Figure 4.18: FISH image of the inoculum biomass sample at day 36 from the SBR2 dye-fed operated with a static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) of the operation 5 at 1000 x. The image shows bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe Gam42a (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

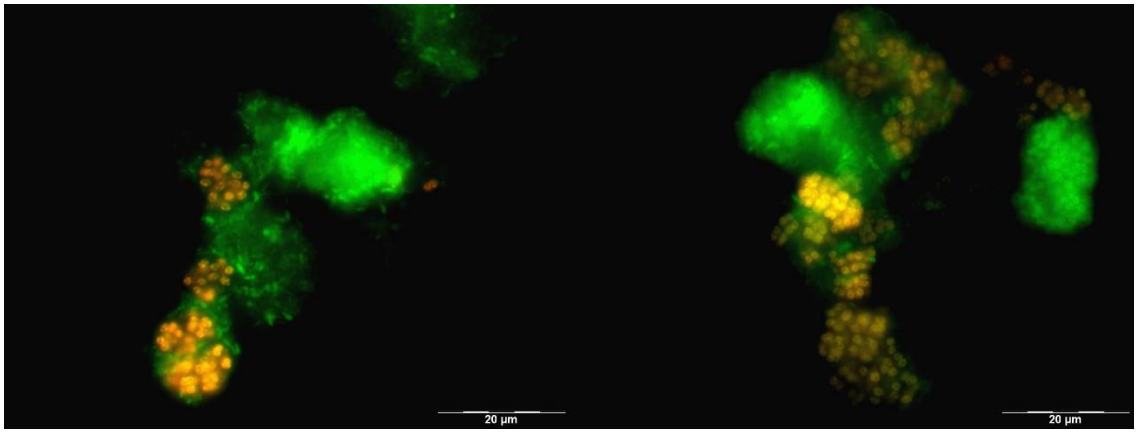


Figure 4.19: FISH images of the biomass samples at days 36 (left) and 52 (right) from the SBR2 dye-fed operated with a static fill followed by a mixed anaerobic phase (1.5 h) and an aerobic phase (3.5 h) of the operation 5 at 1000 x. The images show bacteria hybridized with (FITC)-labelled EUBmix probe (green) and (Cy3)-labelled probe SuperDFmix (red). Bacteria targeting both probes simultaneously result in a yellowish color, whereas other bacteria appear green.

The system used in the fifth operation is described on section 3.1.2. SBR2 was used as control, SBR1 was the one used to study the effect of nanoparticles in the granulation process (both with 1.5 h of anaerobic mixing and 3.5 h of aerobic phase) and the SBR3 was carried out to test the influence of the hydrodynamic regime in granulation.

First, according to the images obtained, FISH allowed the identification of different bacterial populations and it was observed that probes covered a significant part of the total microbial community. The (Cy3)-labelled probes used to select the proteobacterial major groups *Betaproteobacteria* and *Gammaproteobacteria* revealed the highly abundance of these populations in the inoculum sample. However, the abundance of both decreased from the first to the second day of operation analyzed except for SBR1, regarding *Betaproteobacteria* group.

Changes were detected in the decolorization process in the SBR2. From day 8 to 22 it happened a decreased in the percentage of color removal (from 80% to 45%) and after that period the percentage has recovered to 80% again at day 36. As can be seen in **Figure 4.16**, this event was associated to differences in the microbial community. The abundance of the major proteobacterial groups *Alphaproteobacteria* and *Betaproteobacteria* increased whereas *Gammaproteobacteria* populations almost disappeared at day 22. The emergence of a cocci form *Gammaproteobacteria* at day 36 it was observed in parallel to the recovery of the 80% color removal (**Table 3.3**), however, since at day 8 it was not detected a bacterial type with that morphology in abundance, it seems reasonable to affirm the microbial redundancy of the community regarding color removal.

Since the 30th day of operation it was detected amine mineralization only in SBR2. Samples taken at day 36 from the three reactors were used for FISH analysis in order to evaluate if there were evidences of microbial populations implicated in the process. The mixed microbial community differed among the three SBR, *Gammaproteobacteria* was not a relevant group within

the SBR1 and SBR3. However, together with Alf969 and CF319a, Gam42a probes represented a relevant part of the SBR2 biomass sample.

Following the findings achieved in operation 2, the abundance of *Defluvicoccus vanus* related GAOs within the *Alphaproteobacteria* group increased from day 8 to 36 which is associated to the appearance of the amine degradation. However, despite the high abundance of *D. vanus* until day 52, the mineralization of the aromatic amine 4A1NS was only detected until day 43. Interestingly, the disappearance of amine biodegradation happened after cycles with aeration failure (5 days), as expected. However, the microbial community did not recover the amine mineralization capacity. The remained presence of the *D. vanus* related GAOs was expected since it is known that *D. vanus* related GAOs are capable to storage carbon in anaerobic conditions to use in further aerobic conditions, which justify their abundance even after the anaerobic periods occurred. Probably, the mineralization is an enzymatic dependent reaction and the enzymes implicated in amine mineralization were lost during the anaerobic period occurred in some cycles during 5 days and were not recovered when the aeration was replaced.

Regarding SBR1, from day 36 to 106 there was only a slight decrease in the abundance of Betaproteobacteria and it was also observed by FISH ae high abundance of *Citophaga-Flavobacteria*. However, although the performance from SBR1 to SBR2 was similar the microbial community accomplished was different, which is an indication of the microbial redundancy.

On other hand, the major proteobacterial group *Alphaproteobacteria* was the most abundant in SBR3, and also, HGC69a and CF319a (Cy3)-labelled probes represented abundant communities.

Comparing the FISH images obtained from analyzing the sample taken at day 36 from SBR1 and from the control SBR2, it can be noted that the presence of AgNPs affected the microbial community. However, considering SBR1, despite slight differences regarding abundance, the culture remains stable from day 36 until the last day while the microbial community of the remaining reactors had some changes.

In addition, it can be also noticed that the hydrodynamic regime of plug-flow feeding influenced the communities present although, the selected (Cy3)-labelled probes did not cover all the bacterial sample. Furthermore, in SBR3, from day 36 to day 106, it was observed a significant change within the community probably due to some occurrences in the reactor operation, such as lack of suitable feeding of carbon and nitrogen source for long periods.

4.2 Detection and measurement of AHL in aerobic granular sludge

4.2.1 Qualitative approach

The method described in 3.3.2 was applied to qualitatively assess the production of AHL in the reactors during operation 5. The biomass was spotted intact in the plate assay and in the presence of AHL it was expected to observe a change of color of the LB agar plate to blue, starting from the center where the biomass was spotted.

Regarding the results, a difference was noted in the medium color between the control *Agrobacterium tumefaciens* NTL4 and the monitor strain *A. tumefaciens* NTL4 (pZLR4), which suggests the presence of AHL molecules and their successful detection by the monitor strain.

However, a change in the biomass sample color was also detected with both the control and the monitor strains, possibly due to the presence of a microbial community in the biomass sample capable of degrading X-Gal. In addition, a well-defined induced zone was not detected, since all the plate assays presented a slight blue color instead of the expected blue circle. This could be related to the incubation time. Probably a lower incubation time was required in order to correlate the AHL concentration with the blue zone diffused from the centre.

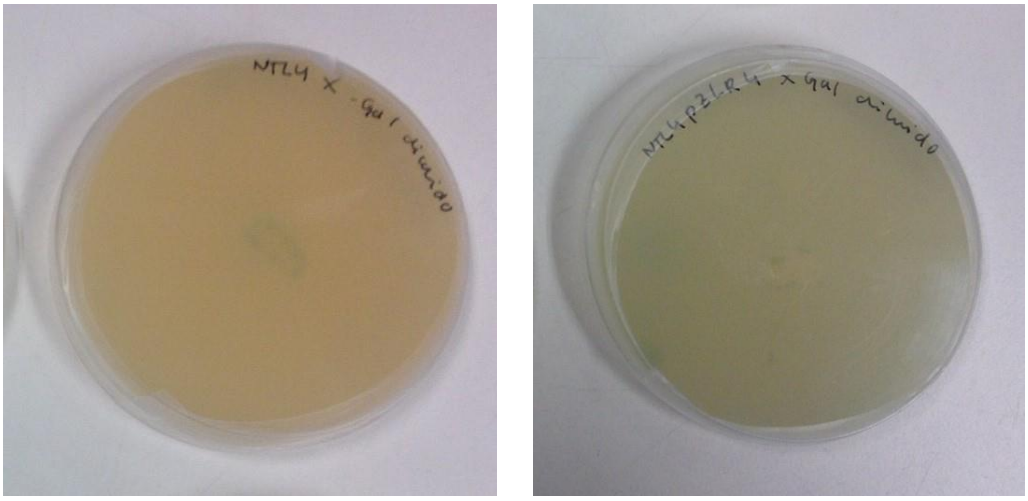


Figure 4.20: *A. tumefaciens* diffusion assays in LB agar. Control strain NTL4 (left) and reporter strain NTL4 (pZLR4) (right). Gently washed biomass samples from SBR2 from the operation 5 were added to the center of the plate.

The present method has some limitations considering AHL quantification. In this sense, it was performed the method described in section 3.3.3.

4.2.2 Quantitative approach

4.2.2.1 Method optimization for AHL measurement

The first step was to establish a calibration curve in order to associate AHL concentrations to the OD_{615} values registered for the blue pigment. At first, the protocol followed was the one described in [8], which is similar to the final method described in 3.3.2.4 section, although with some differences in amounts and in the final step before the optical density reading.

As described in [8], the final volume used in the first experiment was 250 µL, comprehending 100 µL of ODHL solution, 100 µL of the monitor strain *A. tumefaciens* NTL4 (pZLR4) culture (at an OD_{600} of 0.1) and 50 µL of X-Gal (20 mg/mL in DMSO). The method was developed in Eppendorfs regarding the volume scale used. First, we added N-(3-oxo-dodecanoyl)-L-homoserine lactone (ODHL) concentrations between 0 nM and 37.5 nM. The range of concentrations was chosen according to the description of an octanamide AHL detected at 0.15 nM by *A. tumefaciens* [33].

After the cultivation of the monitor strain with the ODHL solution and the addition of X-Gal, an apparent blue color gradation was observed from the blank (blue color was not detected as expected) to the maximum concentration used, which indicated the detection of ODHL by the monitor strain, and also the successful application of the concentrations range selected since they were above the detectable level.

The protocol [8] described a centrifugation step at 10 000 rpm for 10 min which, when performed, resulted in cell sedimentation and seemed to occur also the blue color sedimentation. Therefore, since the AHL measurement will rely on blue pigment concentration, the samples were resuspended with a vortex and were centrifuged again at less aggressive conditions (for 5 min at 4 000 rpm) in order to overcome the blue color sedimentation.

However, despite the attenuation in centrifugation conditions, it was observed once more a blue pellet at the bottom of the Eppendorfs. Before changing again the parameters of the centrifugation, the OD_{615} of each sample was measured in duplicate in a 96-wells plate. The aim was to understand what was happening with the suspension, regarding the results obtained in terms of optical density.

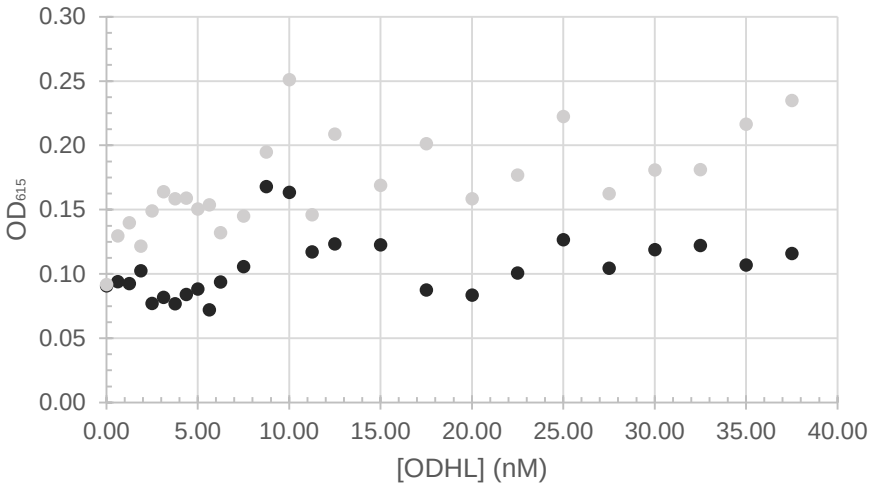


Figure 4.21: 1st attempt to established a relationship between ODHL concentration in nM and optical density at 615 nm. (●) 1st reading; (●) 2nd reading.

Despite the correspondence of the pattern until 32.5 nM, the absence of similarity between the values of the two readings was probably related to the waiting time between readings. Since there was a centrifugation step before the density measurement, perhaps there was some resuspension from the first to the second reading, which leads to higher values of OD_{615}. In addition, regarding the small volumes that were being handled, pipetting errors could introduce large variability between readings. However, analyzing the two readings separately it may be noted a linear relationship in lower concentrations. Furthermore, the results detected between 2.5 nM and 5.63 nM for the first reading cannot be considered since the OD_{615} measurements revealed values below the control value which compromises all the results in this experiment.

The aim of the centrifugation was to avoid the interference of the suspended cells in the absorbance reading and also to sediment the X-Gal that did not react. Since some problems were observed within the centrifugation step it was studied its influence in the method. In addition, the original protocol [33] does not mention any centrifugation.

The centrifugation time was reduced from 5 to 3 min at 4 000 rpm, and two tests were carried out for the same sample, one with centrifugation before the OD_{615} reading and other without that step. The aim of this experiment was to evaluate the impact of the centrifugation in the absorbance measure.

ODHL concentrations between 0 nM and 8.41 nM were measured in duplicate. After the growth procedure, incubation with the AHL solution and X-Gal addition, the OD of the samples was measured at 615 nm in a 96-wells plate after 20 min of sedimentation (1st test) then, all the samples were resuspended (to simulate the initial suspension) and exposed to the centrifugation in order to detect the OD_{615} values (2nd test).

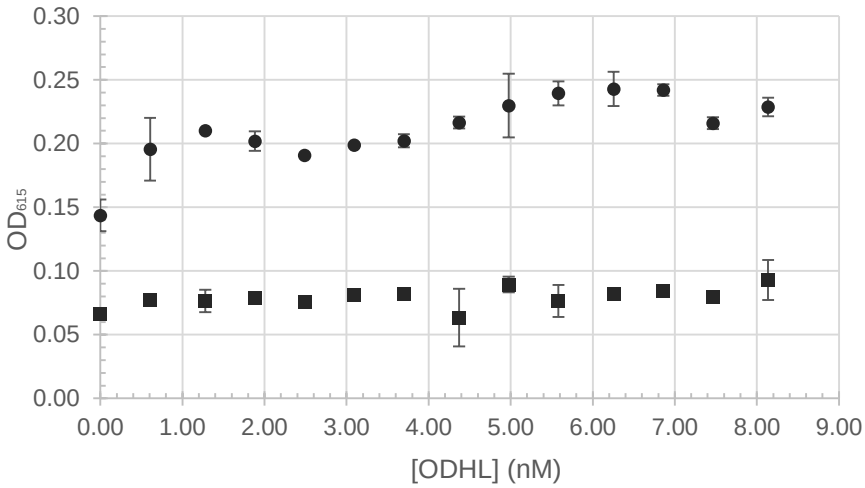


Figure 4.22: Relationship between ODHL concentration in nM and optical density at 615 nm. Comparison between the 1st test performed with 20 min of sedimentation of the samples (●) and the 2nd test performed with a centrifugation step for 3 min at 4 000 rpm both before the OD_{615} measurement (■).

The results showed in **Figure 4.22** revealed a significant signal attenuation when the protocol was carried out with the centrifugation step, even when applied with moderate conditions, which led to the removal of this procedure in the subsequent experiments and to the application of the sedimentation step during 20 min.

Taking into consideration the results previously described, a new assay was attempted in order to obtain the needed relationship between the absorbance and the ODHL concentration (**Figure 4.23**). Both the concentration range and the final volume used were the same tested in the preceding experiment. However, the linearity was not revealed in the conditions used, probably due to the small volumes dealt.

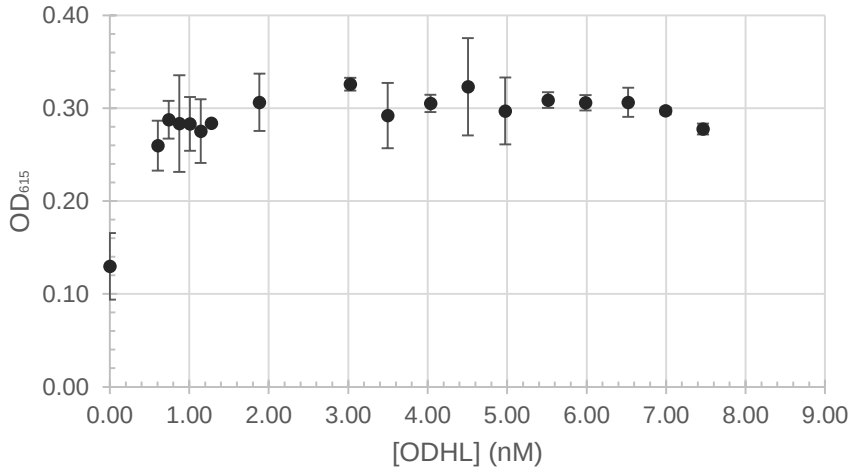


Figure 4.23: (●) Relationship between ODHL concentration in nM and optical density at 615 nm. Each sample was performed with 100 µL of a reporter strain culture (OD_{600} = 0.1) and with 100 µL of an ODHL solution at a known a concentration.

The solution found to overcome this was to increase the final volume of each concentration in test which, despite the challenging handling aspects, is less prone to experimental errors. For instance, small variations in the volume of both the culture and the ODHL solution could interfere in a correct linearity between the absorbance and the ODHL concentration, a wrong agitation and small differences within the conditions applied to all the solutions in the incubation steps could also be a cause for the method failure.

Therefore, rather than the usual 100 µL of culture with an OD_{600} of 0.1 and the same volume of the determined ODHL solution, the first incubation (occurred for 16 to 18 h) was performed with a final volume 10 times higher, 2 mL of the final suspension (which implied 1 mL of culture with an OD_{600} of 0.1 and 1 mL of the ODHL solution). The concentration range of ODHL used in the present experiment was similar to the previous one. Then, regarding the X-Gal addition, 200 µL of each culture was dispensed in Eppendorfs and it was added the described amount of X-Gal (50 µL). The method proceeded with the incubation in darkness and the measurement step was performed as usual.

It was revealed a linear relationship only between 0 nM and 2.00 nM of ODHL (**Figure 4.23**). The linearity found in such low concentrations is mainly due to the specific AHL chosen to do the experiments. ODHL is an AHL type with a particularly complex acyl group which in literature is highly related to low detectable limits by the monitor strain [33]. In addition, the reporter strain used is known as being more sensitive to AHL concentrations when compared to others such as *C. violaceum* [30].

For this reason, a new experiment was conducted with a smaller concentrations range and applying once more, the 2 mL of final incubation volume as in the experiment described before. Therefore, wo linear relationships between ODHL concentrations and OD_{615} were achieved, both with significant linear correlation coefficients (**Figure 4.24** and **Figure 4.25**).

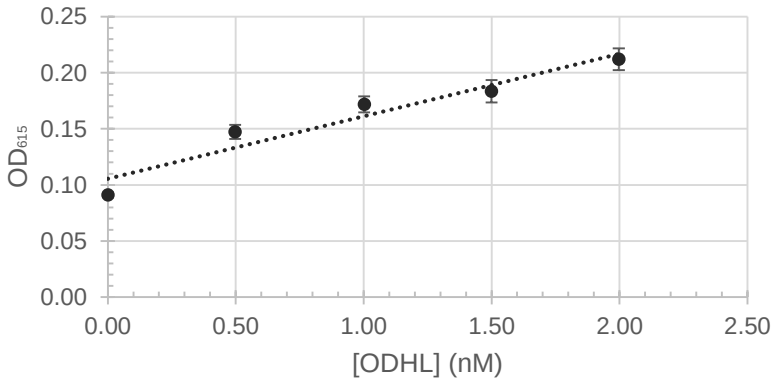


Figure 4.24: (●) Linear relationship between ODHL concentration in nM and optical density at 615 nm, y = 0.0556x + 0.1055, R^2 = 0.9314. Each sample was performed with 10 x higher volumes during the incubation period, 1 mL of a reporter strain culture (OD_{600} = 0.1) and 1 mL of a ODHL solution at a known concentration.

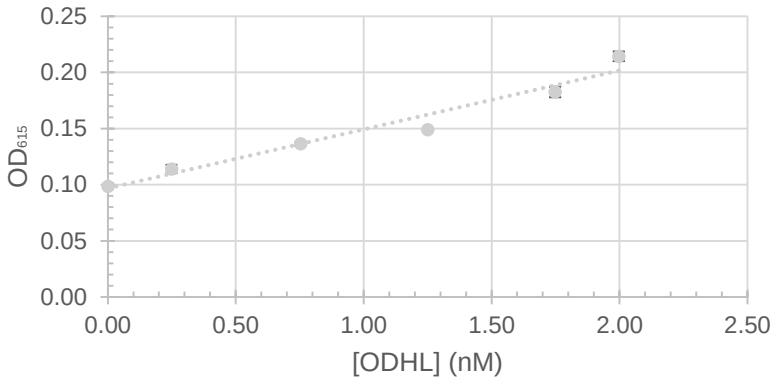


Figure 4.25: (●) Linear relationship between ODHL concentration in nM and optical density at 615 nm, y = 0.0525x + 0.0968, R^2 = 0.9576. Each sample was performed with 10 x higher volumes during the incubation period, 1 mL of a reporter strain culture (OD_{600} = 0.1) and 1 mL of a ODHL solution at a known concentration.

Regarding the standard linear equation y = m·x+b, the strategy followed was to assume the values of the constants as the average of the ones taken from each experiment (**Table 4.1**).

Table 4.1: Constant values of the linear relationship between ODHL concentration and OD_{615} obtained for both the experiments, and the respective average.

	m	**b**	**R^2**
Experiment 1 (Figure 4.24)	0.0556	0.1055	0.9314
Experiment 2 (Figure 4.25)	0.0525	0.0968	0.9576
Average	**0.0541**	**0.1012**	**0.9445**

Which resulted in the following equation.

$$[AHL]\ (nM_{ODHL}) = 0.0541 \cdot OD_{615} + 0.1012 \quad [1]$$

Finally, it is important to empathize the need of increasing the incubation volume to overcome the initial problems as the application of a sedimentation instead the centrifugation step referred in Li et al. (2014) [8]. The method was established at these conditions and the quantification of AHL in AHL extracts was revealed as possible using ODHL as reference. The following analyses were performed considering these alterations to the initial protocol studied [8] and the final protocol is described in section 3.3.3.

4.2.2.2 AHL measurement

After the optimization process that revealed the need of increasing the incubation volumes and the introduction of a settling step instead of the centrifugation (as referred in the previous section), selected samples from days 1 to 52 of the fifth operation were analyzed according to the

method described in 3.3.3.2 in order to quantify the amount of AHL produced during the granulation process and the effect of silver nanoparticles (AgNPs) within this phase.

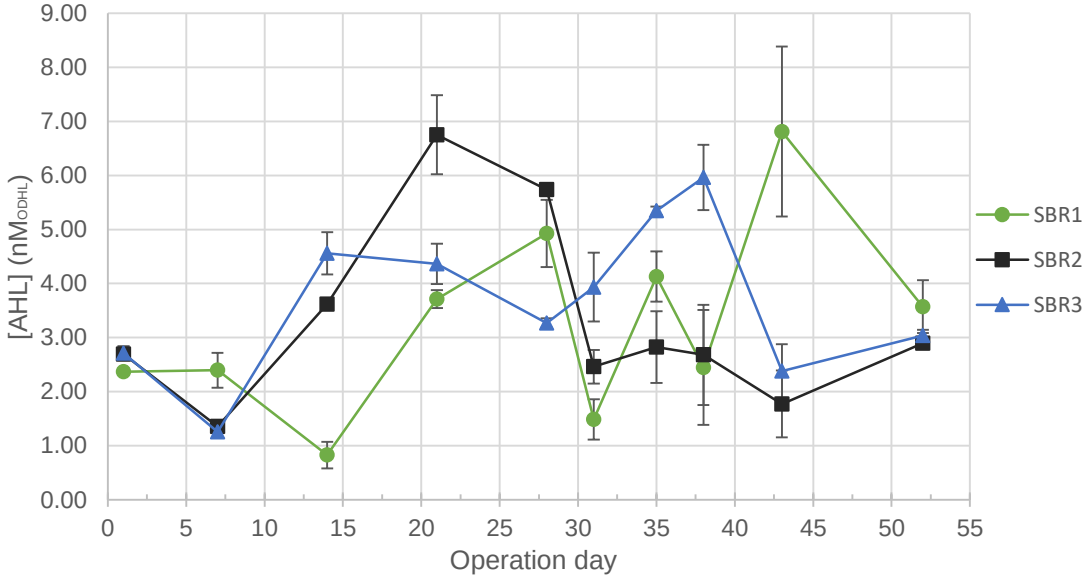


Figure 4.26: Evolution of AHL concentration in nM of ODHL during the operation for the dye-fed mixed with AgNPs bioreactor in mixed anaerobic phase (1.5 h) followed by anaerobic phase (3.5 h)– SBR1 (●), the dye-fed bioreactor in mixed anaerobic phase (1.5 h) and aerobic phase (3.5 h) – SBR2 (■), and for the dye-fed plug-flow feed bioreactor – SBR3 (▲). The concentrations were achieved using the relation [AHL] = 0.0541·OD_{615} + 0.1012, R^2 = 0.9445.

The AHL measurement, for each reactor, was carried out in different days however, the values of the sample control were similar between experiments (0.1135, 0.1198 and 0.1245 on average, respectively for SBR1, SBR2 and SBR3 trials). For each one of the AHL supernatants S2 obtained from the reactors it was performed an incubation with the reporter strain in duplicate and then, the incubation with X-Gal was also performed in duplicate for each sample. After that, the OD_{615} was measured.

Taking into account the relationship between ODHL amounts and OD_{615}, achieved in the previous section, it was possible to obtain specific concentration values at each point of operation.

The results shown in **Figure 4.26** are related to the granulation period in each reactor that can be followed by the sludge volume index (SVI) profile described in **Figure 4.27** It was interesting to observe that in the beginning of the operation, the detected AHL concentrations were similar in SBR2 and SBR3, and that the evolution along 14 days of operation was the same. The produced AHL amounts decreased from day 1 to 8 regarding the adaptation of the microbial community to the environment and conditions imposed by the experiment. However, in SBR1, in which the experiment was carried out with the presence of AgNPs, the adaptation was slower and there were identified similar AHL concentrations during the first week in this specific reactor. Then, the reduction of AHL production was detected in SBR3 as it was detected in the other

reactors which could correspond to an adaptation of the three reactors regarding the conditions imposed.

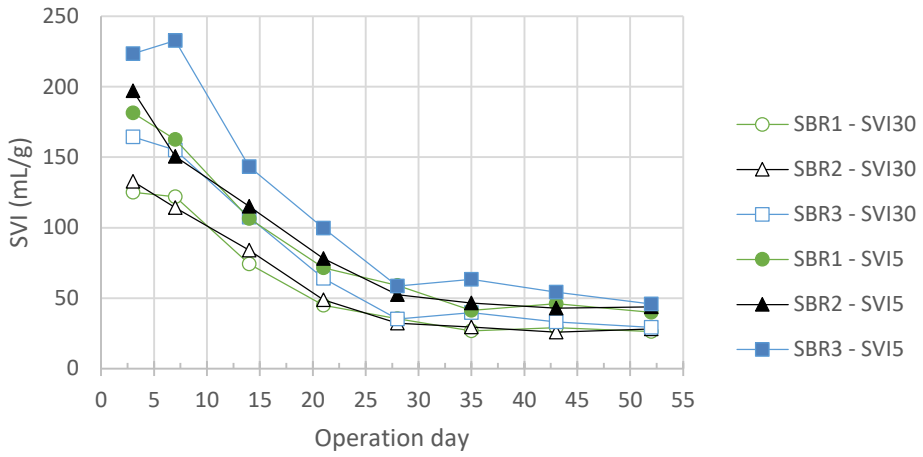


Figure 4.27: Evolution of the sludge volume index at 5 min and at 30 min during the granulation period of the operation 5.

After the adaptation period faced by all the reactors, the AHL measurement revealed an increase trend in the production of AHL. The first peak production occurred at different days for all the reactors (at day 28 for SBR1, at day 21 for SBR2 and at day 14 for SBR3) and the higher value was detected in the control reactor, SBR2.

Despite the similarity observed during the first week of operation in SBR2 and SBR3, the production of AHL started to differentiate, probably due to the distinct hydrodynamic regimes imposed. Since the lowest values for AHL concentration were detected at day 7 (1.35 nM_{ODHL} and 1.26 nM_{ODHL}, respectively in SBR2 and SBR3), the AHL production seemed to increase and this might be associated to the beginning of the aggregation. Furthermore, interestingly the peaks correspond to the days when it was observed proximate and low values of sludge volume index at 5 min (SVI_5) and at 30 min (SVI_{30}), which indicates good granulation characteristics. For SBR2, 78 mL/g and 49 mL/g SVI_5 and SVI_{30}, respectively and for SBR3, 54 mL/g and 33 mL/g, SVI_5 and SVI_{30}, respectively.

After that period, it was detected a reduction in the production of AHL (at day 31 for SBR1 and SBR2 and at day 28 for SBR3) therefore, considering operational aspects this decrease might be associated with the imposition of 5 min settling instead of the decreasing time settling imposed in the first weeks as described in section 3.1.2. The difference observed between SBR1/SBR2 and SBR3 was probably due to their distinct hydrodynamic regimes however, an error has occurred in the aeration of the SBR3 at day 28 that may have influenced the AHL production.

Since the granules were stable at this time for SBR1 and SBR2 (around day 30), the evolution pattern regarding the AHL production needs to be understood. In SBR1 were detected two increases in AHL production, at days 35 and 43, which seemed to divert from the trend in that

period. After analyzed the data from these operational days, it can be concluded that, the values detected corresponded to days when it was observed great biomass loss (1.1 g/L in both of the days), by analyzing the results obtained from the total suspended solids (TSS) test in the effluent (**Table 3.4**).

In addition, the slight increase detected at day 35 in SBR2 was also consistent to biomass losses (1.3 g/L). These findings allowed the assumption that biomass losses induce the production of signaling molecules (such as AHL) in a response to stress in order to promote the granulation.

For SBR3 no significant biomass losses were detected that could explain the increasing production of AHL at day 38 although, once the granulation in this specific reactor had been delayed, the higher production of AHL detected might be partially caused by that granulation. Furthermore, the pronounced AHL decrease detected after the peak observed at day 38 was similar to the evolution detected in the other reactors after the granulation had already ended.

In literature it is compared the AHL production in AGS with the production in flocculent activated sludge (FAS) [8], and the measurement occurred after the granulation phase, where the granules were stable. In addition, the relative AHL content was calculated as the ratio of the absorbance of the sample over the absorbance of the control (fold-induction). That study mentioned values between 2.1 fold-induction and 2.4 fold-induction for AGS. Therefore, in order to compare these results to the ones achieved in this work the same strategy was followed for the sample at day 52 (day after granulation was observed). Interestingly, a higher value was determined than for SBR2 (2.4 fold-induction) and the lower value was detected in the plug-flow feed bioreactor (1.5 fold-induction). These findings confirm the reported results for AGS applied for generic synthetic wastewater using glucose (SBR1) and sodium acetate (SBR2) as carbon source. However, studies regarding AHL quantification has not been reported for AGS and mixed microbial cultures.

5 Conclusions

5.1 Characterization of mixed microbial culture

Taking into account the FISH probes choice, bacteria were the major domain represented in each individual sample, since nearly all the biomass gave positive signal for EUBmix. Moreover, the groups specific probes were representative of the most abundant bacterial groups and a good coverage was achieved in the survey carried out at different operational periods of the AGS reactors analyzed in this study.

At the end of operations 1 and 4 it was detected high abundance of *Gammaproteobacteria*. Therefore, it was discovered that the lack of diversity within the community could be associated with granules fragility. However, since the reactor of the 1st operation was operated in a different aeration regime, the conditions imposed could also had impact on the granules characteristics. In operation 4 it was also detected that after the addition of acetate within the feed it was detected granule dissociation.

Furthermore, considering *Gammaproteobacteria* it was revealed their probably importance in color removal in specific operations however, it was also detected the functional redundancy of the community to remove color, since the replacement of a *Gammaproteobacteria*-dominated community for a more diverse and even community also resulted in good color removal results.

In addition, the plug-flow feed regime promoted an even community allowing the growth of organisms with different substrate affinities and have also importance in smoothing the shock imposed by composition changes within the feed.

Besides, FISH analysis carried out before and after biomass storage allowed the assessment that storage had impact on the mixed microbial culture composition. In particular, it was detected that the A*lphaproteobacteria* group *D. vanus* related GAOs is highly susceptible to long term storage periods at 4 ºC.

Following this finding, it could also be hypothesized that the presence of *D. vanus* related GAOs might be related to the biodegradation of the secondary amine resulting from dye degradation. This hypothesis is based on the fact that it was detected amine mineralization at the end of the 2nd operation and then, that microbial culture's capacity disappeared during the storage period. In addition, the amine biodegradation arose again at operation 4 and the FISH experiments allowed the identification of *D. vanus* related GAOs also during the days when that biodegradation occurred. However, despite the presence of *D. vanus* related GAOs within the biomass sample, after an unplanned anaerobic period, the desired capacity disappeared again.

5.2 Effect of AgNPs and of the SBR hydrodynamic regime in the granulation process

The aim of understanding the effect of AgNPs presence in the granulation process and also to study the effect of the hydrodynamic regime in this process was successfully achieved.

The method used to detect the presence of AHL within the reactors proved to be poorly sensitive and liable to errors regarding the detection and measurement of the blue zones formed around the biomass due to X-gal hydrolysis. Therefore, alterations to the method were introduced in order to improve the sensitivity, as well as to determine a quantitative estimate of the AHL content in the reactor, and to establish the relationship between granulation capacity and QS. In order to achieve quantitative values of concentration, a synthetic AHL (ODHL) was used as standard. It was necessary an optimization regarding the original method described for this purpose. After the linear relationship had been reached, the reactors samples were tested.

The biomass adaptation to the conditions imposed in the reactors without AgNPs in the feed was similar. However, the adaptation in the reactor fed with AgNPs took longer and the decrease of AHL production, which is associated with an adaptation to the changing environment, was delayed for a week. These findings could indicate the effect of AgNPs in the granulation phase. The increase in AHL production detected later in the three reactors matched with the beginning of the granulation. After the adaptation, it was in SBR2 that the AHL concentration was higher when compared to the reactor containing AgNPs (SBR1), suggesting that these nanoparticles may negatively interfere with the granulation process. It was in this period that the hydrodynamic regime imposed assumed an important role since the evolution of AHL production was higher in SBR2 than in SBR3.

It has also been discovered an association between biomass losses and the increase in AHL production. Biomass losses probably induce cell stress leading to an increase in AHL production in order to promote the granulation and allowing the permanence within the reactor.

5.3 Future work

In microbial ecology aspects, the broad study performed in this master thesis should be supported by high throughput sequencing results or with quantitative FISH to complete and detail further the mixed microbial community characterization. In addition, it could also be interesting assess the presence of other microorganisms using for instance, the fluorescence stain DAPI. And finally, study bacterial groups within the proteobacterial groups used in this work.

The QS studies could offer a better understanding of the granular structure, and this point is particularly interesting since, AGS has been reported as the next generation of wastewater treatments and comprehending the structure is important to engineering control. Along with the study of the AHL production during the granulation process it could also be relevant to relate the QS molecules to granules stability and response to stress. Regarding the method used, it could

be interesting identify the AHL molecules produced during the operation since reporter strains are sensible to different kind of signaling molecules.

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Annexes

A. Growth curves for *Agrobacterium tumefaciens* NTL4 and NTL4 (pZLR4)

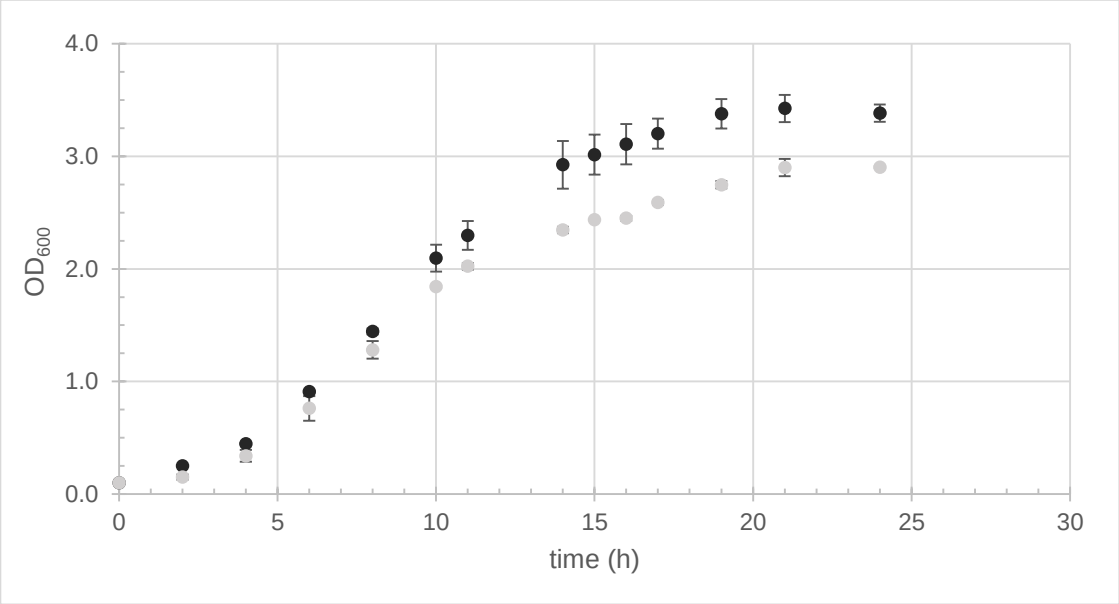

Figure: A.1: Growth curve for *A. tumefaciens* NTL4 (●) and *A. tumefaciens* NTL4 (●) harboring pZLR4. Growth was performed in Erlenmeyer flasks at 28 °C and 150 rpm in liquid LB medium, for strain the NTL4 (pZLR4) supplemented with gentamicin (50 µg/mL).