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Removal of Escherichia coli as indicator of wastewater pathogens: a comparison between Aerobic Granular Sludge and Activated Sludge laboratory reactors

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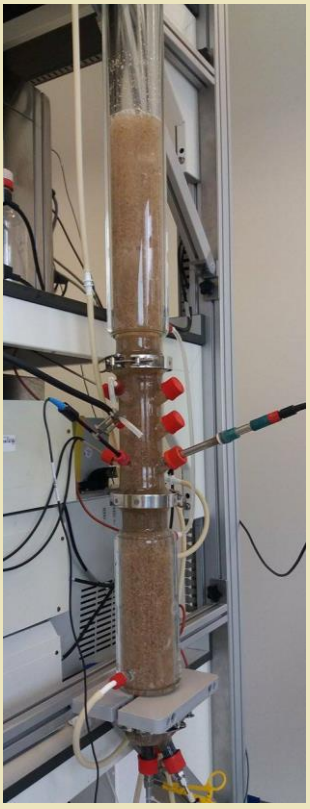

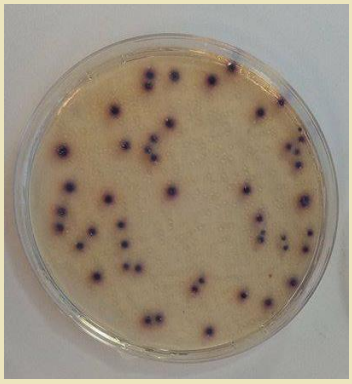

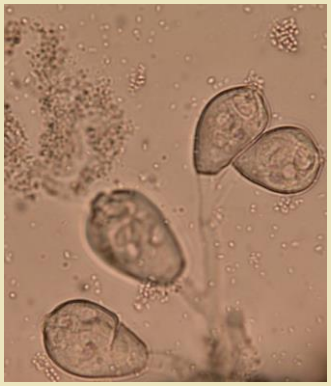

Removal of Escherichia coli as indicator of wastewater pathogens: a comparison between Aerobic Granular Sludge and Activated Sludge laboratory reactors

Master of Science Thesis

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Abstract

An Aerobic Granular Sludge (AGS) and an Activated Sludge (AS) laboratory-scale reactors started-up with seed sludge originated from the same wastewater, and fed with the same synthetic wastewater containing *Escherichia coli*, were operated and weekly sampled during four months in order to compare the removal of *Escherichia c*oli between both set-ups.

Both reactors were aimed to remove organic matter, nitrogen, and phosphate. The removal of organic matter and phosphate was successful, whereas nitrogen removal efficiency fluctuated in the AGS reactor, and was never achieved in the AS reactor. In order to enhance nitrification, several changes were introduced in the AS cycle, whilst the AGS cycle was maintained unchanged during the entire research period, only varying DO concentrations.

E. coli was added to the reactors after 1.5 months of operation, and weekly samples were analysed with the spread-plate technique. The log removal of *E. coli* for the AGS reactor was above 1.5 for the first five weeks of *E. coli* addition, presenting a maximum of 3.5 log removal and an average of 2.5 log removal. After the fifth week, the removal dropped to zero, and started to slowly increase again, achieving 2.5 log removal by the end of the research period.

The removal of *E. coli* was mainly attributed to predation of *E. coli* cells by stalked ciliates of the genus *Vorticella* during aeration. *Vorticella* were observed by optical microscopy attached to granules, in high amounts during the periods of high *E. coli* removal, and in lower amounts when *E. coli* removal decreased. The factors affecting the fluctuations in the presence of ciliates were analysed, finding as the most relevant an event of high copper concentration and the possible depletion of particulate food eroded from the granules. Analysing the concentration of *E. coli* in the sludge and supernatant, it was deducted that there was attachment of some *E. coli* cells to the granules. When *E. coli* removal was significant, on average, 26% of the influent *E. coli* ended-up in the sludge and 0.2% in the effluent.

E. coli removal in the AS reactor was never achieved. Despite AGS and AS reactors were started with seed sludge originated from the same influent wastewater, ciliates were not detected in the AS reactor on samples taken after two months of operation onwards. It was assumed that the main cause of the absence of ciliates was the lack of particulate food. Attachment of *E. coli* cells to bacterial flocs was not detected neither, this might be attributed to the high SRT of the reactor.

A basic analysis of the applicability of AGS treatment in two case studies in Uruguay was performed, finding that the technology would be in principle suitable for the treatment of the wastewater of both localities aiming to comply the Uruguayan standards. In the case of Mercedes, it seems that an AGS WWTP would result in a more cost-effective solution than a conventional treatment. However, a pre-design and cost analysis should be performed to confirm this hypothesis. Montevideo's wastewater has a high conductivity, and despite according to lab-scale research, AGS performance would not be affected by its level of salinity, the suitability of the technology for Montevideo's wastewater would only be confirmed after AGS is proven to be successful in treating wastewater with these characteristics.

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Abbreviations

AEB	End anaerobic
AEE	End aerobic
AGS	Aerobic Granular Sludge
ANAMMOX	Anaerobic Ammonium Oxidation
AS	Activated Sludge
BOD	Biochemical Oxygen Demand
CFU	Colony Forming Units
COD	Chemical Oxygen Demand
CSO	Combined Sewer Overflow
DGGE	Denaturing Gradient Gel Electrophoresis
DINAMA	National Environmental Authority of Uruguay
DO	Dissolved Oxygen
DS	Dry Solids
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EPA	Environmental Protection Agency
EPS	Extracellular Polymeric Substances
EU	European Union
FISH	Fluorescent In Situ Hybridization
GAO	Glycogen Accumulating Organisms
HRT	Hydraulic Retention time
ISO	International Standardization Organization
MLSS	Mixed Liquor Suspended Solids
MPN	Most Probable Number
M.Sc.	Master of Science
MVOTMA	Ministry of housing, land use, and environment of Uruguay
NEN	Dutch Standards Institute
OSE	National Water Supply and Sanitation Company of Uruguay

PAO	Polyphosphate Accumulating Organisms
PE	Person Equivalent
PFU	Plaque Forming Units
(q)PCR	(Quantitative) Polymerase Chain Reaction
RNA	Ribonucleic Acid
RWF	Rain Weather Flow
SBR	Sequencing Batch Reactor
SEM	Scanning Electron Microscope
SRT	Solids Retention time
STOWA	Dutch Foundation for Applied Water Research
STW	Dutch Technology Foundation
SVI	Sludge Volume Index
TEM	Transmission Electron Microscopy
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TOD	Total Oxygen Demand
TP	Total Phosphorus
TSS	Total Suspended Solids
TU Delft	Delft University of Technology
VSS	Volatile Suspended Solids
WAS	Waste Activated Sludge
WWT(P)	Wastewater Treatment (Plant)

CHAPTER 1

Introduction

In the framework of a Master of Science thesis in Sanitary Engineering at UNESCO-IHE, a research was conducted to study the removal efficiencies, removal mechanisms, and fate of *Escherichia coli* in the novel Aerobic Granular Sludge treatment technology in comparison with the well-known Activated Sludge treatment technology. In this way, two laboratory-scale reactors subjected to the same conditions and fed with the same synthetic wastewater were studied: one Aerobic Granular Sludge reactor at Delft University of Technology laboratories and one Activated Sludge reactor at UNESCO-IHE laboratories. Furthermore, the applicability in Uruguay of this novel treatment technology was studied.

This document describes the aforementioned M.Sc. thesis research. In Chapter 1, background, problem statement and justification, and research questions and objectives, are presented. In Chapter 2, a summary of the current literature regarding topics of relevance for this research is presented. In Chapter 3, the materials and methods for the conduction of this research are detailed. The results and their discussion are presented in Chapter 4. Finally, in Chapter 5, the conclusions of the research and recommendations for further studies are given. Additional information is presented in annexes.

1.1. Background

One of the major threats to public health in developing countries is the transmission of pathogens by the faecal–oral route. Domestic wastewater contains a myriad of pathogens, and therefore, if it is not appropriately disposed, it might be a source of faecal–oral contamination.

Conventional wastewater treatment technologies, such as Activated Sludge (AS), are known to remove a fraction of the pathogens present in the influent as it will be presented in Section 2.4 of this document. Nevertheless, this removal efficiency could be insufficient in some cases, such as when the effluent is discharged in water courses with recreational uses, or when it needs to be reused due to water scarcity, a situation which is becoming worse every day because of climate change, population, and urbanization growth. The majority of the removed pathogens end up in the sludge, and therefore also a careful handling of the sludge should be ensured to avoid human contamination in case of sludge reuse. The level of risk to human health associated with these cases depends on the final concentration of pathogens in the effluent and in the sludge.

Aerobic Granular sludge (AGS) is a treatment technology similar to AS in some aspects, which has been deeply studied at laboratory-scale in the last twenty years, but not much applied in

full-scale yet. It is a more cost-effective technology than AS because of its smaller footprint and significant pumping energy savings. As it is a novel technology, there are still topics which have not been yet studied and need to be addressed.

In order to study the removal of pathogens in wastewater treatment systems, it is common to use indicator organisms instead of pathogens, because their analysis is simpler, more cost-effective, and safer; a review on this topic is presented in Section 2.3.

1.2. Problem statement

The removal efficiencies and fate of pathogens in wastewater treatment technologies should be known in order to assess the risk to public health associated with the disposal of treated wastewater or with the handling of the sludge. AS is a traditional treatment technology, which has been much studied, including research on the removal efficiencies, removal mechanisms, and fate of pathogens.

In 2004 the first AGS workshop was held in Munich, congregating researchers from all over the world which were investigating this treatment technology. During the workshop discussion, one of the research topics which emerged as not yet revealed is the way in which pathogens are removed by AGS in comparison to AS (Bathe, et al., 2005).

Due to the differences between aerobic granules and AS flocs, regarding their density and their interaction with suspended solids, AGS pathogen removal capacity is expected to be different from that from AS (De Kreuk, 2006). De Kreuk (2006) concluded that pilot plant research should be done in this matter in order to protect public health.

To date, the study of pathogens' removal and fate in AGS is still not reported in literature, and therefore it is identified as a knowledge gap which requires further investigation.

1.3. Justification

AGS is a treatment technology which is being applied worldwide at full-scale since 2009, when the first full-scale demonstration AGS system for domestic wastewater was installed as an upgrade of Gansbaai Wastewater Treatment Plant (WWTP) in South Africa (Water technology, 2012). After this experience, a plethora of other full-scale plants were installed around the world in countries such as the Netherlands, Poland, Ireland, Brazil, South Africa and Portugal; and many other projects are planned also for the Netherlands, Australia, Brazil, Ireland, Portugal, South Africa, Sweden, Switzerland, and United Kingdom (Royal Haskoning DHV, 2016).

AGS is a treatment technology which achieves good effluent quality at low cost due to its small footprint and high energy savings (De Bruin, 2013). Compared to conventional technologies, such as AS, the footprint can be reduced four times (De Bruin, et al., 2004), and the energy savings might reach 20% (De Bruin, 2013).

Many authors agree that AGS is a promising technology in the near future, as shown in the following statements:

''Aerobic granular sludge is a very promising technology from an engineering as well as economic point of view, and should therefore be further developed.'' (De Bruin, et al., 2004)

''To date, the application of aerobic granular sludge was regarded as one of the promising biotechnologies in wastewater treatment.'' (Adav, et al., 2008)

''Based on the growing interest from The Netherlands and from abroad a fast (international) rollout of the technology is expected.'' (De Bruin, 2013)

This leads to consider that AGS is a trend in wastewater treatment technologies and probably many other full-scale systems are to be installed all over the world in the coming years, including developing countries, where cost-effective technologies are welcomed. In these countries the need for water reuse is common and the risk of faecal-oral contamination is very high if wastewater and sludge are not carefully managed. These facts support the need for studying the removal of pathogens in AGS and also for evaluating the possibilities of applying this treatment technology to countries which have difficulties in funding the treatment of their domestic wastewater, such as Uruguay in South America.

1.4. Research questions

As mentioned before, indicator organisms are commonly used to study the removal of pathogens in wastewater treatment. Therefore, in order to start filling the gap regarding the lack of studies about the removal of pathogens in AGS, the following research questions focusing on the indicator organism *Escherichia coli* in laboratory set-ups were developed.

- What are the removal efficiencies of *E. coli* under AGS treatment conditions?
- What are the *E. coli* removal mechanisms under AGS treatment conditions, and which one is dominant?
- Which proportion of influent *E. coli* ends up in the effluent and which proportion in the sludge?
- Is there a difference in removal efficiencies, removal mechanisms, and fate of *E. coli* in AGS compared to AS?

To answer these questions, which emerged from the problem statement, a set of objectives were established. These are presented in the following section.

1.5. Research objectives

1.5.1. *Main objectives*

The aim of this study is to determine the removal efficiencies, removal mechanisms, and fate of *E. coli* in an AGS and in an AS laboratory-scale reactors fed with synthetic wastewater. Furthermore, the applicability of AGS treatment technology in Uruguay is evaluated.

1.5.2. *Specific objectives*

The specific objectives which guided the research to achieve the main objectives are the following.

1. To determine and compare the removal efficiencies, removal mechanisms, and fate of *Escherichia coli* in an AGS and in an AS laboratory-scale reactors subjected to the same conditions and fed with the same synthetic wastewater.
2. To determine the influence of the variation of some conditions of the reactor, on the removal efficiencies, removal mechanisms, and fate of *E. coli* in an AGS laboratory-scale reactor.
3. To evaluate the applicability of AGS treatment technology for domestic wastewater in Uruguay by comparing the characteristics of the wastewater with cases around the world in which AGS treatment technology is already successfully applied at full-scale level.

The first two objectives are in the framework of the Ph.D. research of M.Sc. Mary Luz Barrios at UNESCO-IHE.

CHAPTER 2

Literature review

In this chapter, a summary of the most relevant literature related to the topics under study is presented.

2.1. AGS treatment technology

2.1.1. *History and present situation of AGS*

Granular sludge was first discovered in anaerobic industrial wastewater treatment systems in the late 1970s (Gao, et al., 2011), at Wageningen University (Winkler, 2012). According to De Kreuk, et al. (2007), twenty years later, AGS research started, growing aerobic granules in a Sequencing Batch Reactor (SBR) without carrier material, fed with readily biodegradable substrate. This was performed between Morgenroth, et al. (1997) (Technical University of Munich), and Van Loosdrecht and Heijnen (1993) (Delft University of Technology). These first researches were accomplished with the support of the Dutch Technology Foundation (STW) (De Bruin, 2013). According to Winkler (2012), Ph.D. student Janneke Beun proved that COD and nitrogen could be removed in a sequencing batch airlift reactor discontinuously fed, while the granules stayed stable, leading to the submission and grant of an international patent in 1998. Later, Merle de Kreuk included an extended anaerobic feeding period to the process, achieving simultaneous COD, nitrogen, and phosphorus removal, and stable granular sludge formation enhanced by slow growing organisms selection (Winkler, 2012).

According to De Bruin (2013), around the year 2000, the Dutch water sector became interested in AGS treatment technology and thus, Dutch Foundation for Applied Water Research (STOWA) started a pilot research project in Ede WWTP, the Netherlands. They also point out that in the year 2005 Delft University of Technology, STOWA, and Royal HaskoningDHV, as a private-public partnership, initiated the programme National Nereda Research Programme aimed to develop Nereda technology for the removal of COD, nitrogen, and phosphorus; subsequently six Dutch Water Boards joined the programme. After Ede, many other pilot plants were installed around the Netherlands in the period 2006 - 2010 with successful results (De Bruin, 2013).

The first full-scale demonstration AGS system for domestic wastewater was applied in 2009 in Gansbaai, South Africa, for the upgrade of the current wastewater treatment plant (WWTP) (Water technology, 2012). After the successful results of Gansbaai WWTP, the first newly built full-scale AGS plant for municipal wastewater was installed in Epe, the Netherlands in 2012 (Water technology, 2012). Royal Haskoning DHV (2016) announces that including Epe plant, three full-scale treatment plants were installed in the Netherlands for domestic wastewater

and many others were installed in countries such as South Africa, Ireland, Poland, Brazil, and Portugal (two WWTPs were installed in the first two countries and one in the latter ones). Furthermore, they announce that at present, there are two more projects located in the Netherlands, in Simpelveld and in Utrecht, and a plethora of other projects in Australia, Brazil, Ireland, Portugal, South Africa, Sweden, Switzerland, and United Kingdom. In the following paragraphs, short descriptions of the first full-scale AGS WWTPs Gansbaai and Epe, and of Garmerwolde WWTP are given; these plants were chosen to be described as there is a plethora of accessible information about them.

Gansbaai WWTP

Gansbaai WWTP is located in the Overstrand municipality of South Africa in the Western Cape. The upgrade of the plant, which incorporated AGS technology, was commissioned in 2009 by SSI, a South African engineering and environmental consultancy company, subsidiary of Royal HaskoningDHV (Kolver, 2012).

Royal Haskoning DHV (2016) states that the plant was designed for an average flow of 5,000 m^3/d, a peak flow of 400 m^3/h, and 63,000 PE. They also announce that compared to conventional technologies, this plant was 60% smaller and presented a reduction in capital costs of 40%. In the following table, the performance data of the treatment plant for the year 2011 is presented. In Figure 2-1 an aerial view of the plant is shown.

Table 2-1: Performance data of Gansbaai WWTP – 2011. Adapted from Giesen and Thompson (2013)

Parameter	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
COD total	1,265	40	97
TKN	115	---	---
NH_4-N	75	< 1	> 98
TN	---	< 10	89
TP	19	3.2	82
TSS	450	< 5	99

Figure 2-1: Aerial view of Gansbaai WWTP (Dutch water sector, 2015)

Epe WWTP

Epe WWTP is situated in the homonymous locality, in the Netherlands. It was designed by Royal HaskoningDHV and it is in full operation since January 2012 (Dutch water sector, 2013), by Dutch Water Board Waterschap Veluwe (Water technology, 2012). Royal Haskoning DHV (2016) states that the plant was designed for an average flow of 8,000 m^3/d, a peak flow of 1,500 m^3/h, and 54,000 PE (based on 1 PE = 150 g TOD). According to Water technology (2012), it treats domestic and industrial wastewater, with a contribution of 15% from slaughterhouses.

According to De Bruin (2013), due to the large slaughterhouse wastewater contribution, the plant has an extensive pre-treatment consisting of perforated screens and sand/fat removal. They state that after the pre-treatment there are three Nereda reactors designed for an MLSS concentration of 8 g/L and a sludge loading of 0.12 kg COD/(kg DS.day). As post-treatment there is a discontinuous sand filtration with alum dose; De Bruin (2013) justified the inclusion of this unit due to the limited experience on post-treatment with simultaneous residual-phosphorus and suspended solids removal. They explained that the backwash water from the filters together with the waste sludge of the Nereda reactors (which is accumulated first in a buffer tank) go to belt thickeners, reaching a dry solids concentration of more than 5.5% with 1 – 2 g/kg DS of polymer dose. The thickened sludge is stored in a buffer tank and then transported to Apeldoorn WWTP for digestion (De Bruin, 2013).

The effluent of the WWTP complies with the stringent standards for nitrogen and phosphorus of 5 to 8 mg/L and 0.3 to 0.5 mg/L respectively (De Bruin, 2013). According to the same author, while Epe consumes 21.2 kWh/PE/year, on average, a conventional WWTPs in the Netherlands consumes 37.5 kWh/PE/year, this means 40% of energy savings.

In the following table, the performance data of the treatment plant for the year 2012 is presented.

Table 2-2: Performance data of Epe WWTP – 2012. Adapted from Giesen and Thompson (2013)

Parameter	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
COD total	879	27	97
TKN	77	1.4	98
NH_4-N	54	0.1	100
TN	---	< 4.0	---
TP	9.3	0.3	97
TSS	341	< 0.5	100

Garmerwolde WWTP

Garmerwolde WWTP is located near Groningen, the Netherlands. It is operated by Dutch Water Board Noorderzijlvest, and according to Pronk, et al. (2015) it is one of the largest full-scale AGS plants for domestic wastewater. They also point out that originally, the WWTP consisted only of an AS system, but it was decided to upgrade it with the inclusion of an AGS system in order to meet the effluent requirements. They state that the AGS system was designed by Royal HaskoningDHV, and it is in full operation since July 2013. Royal Haskoning DHV (2016) indicates that the AGS plant was designed for an average flow of 30,000 m^3/d, a peak flow of 4,200 m^3/h, and 140,000 PE. According to Pronk, et al. (2015), this average flow corresponds to 41% of the total influent to the WWTP. They also mention that the energy consumption of this AGS plant is 58 to 63% lower than an average AS WWTP in the Netherlands. An aerial view of the plant and a process scheme of the treatment are depicted in the following figures (Pronk, et al., 2015).

Figure 2-2: Aerial view of Garmerwolde WWTP. AGS plant is located in the front (Pronk, et al., 2015)

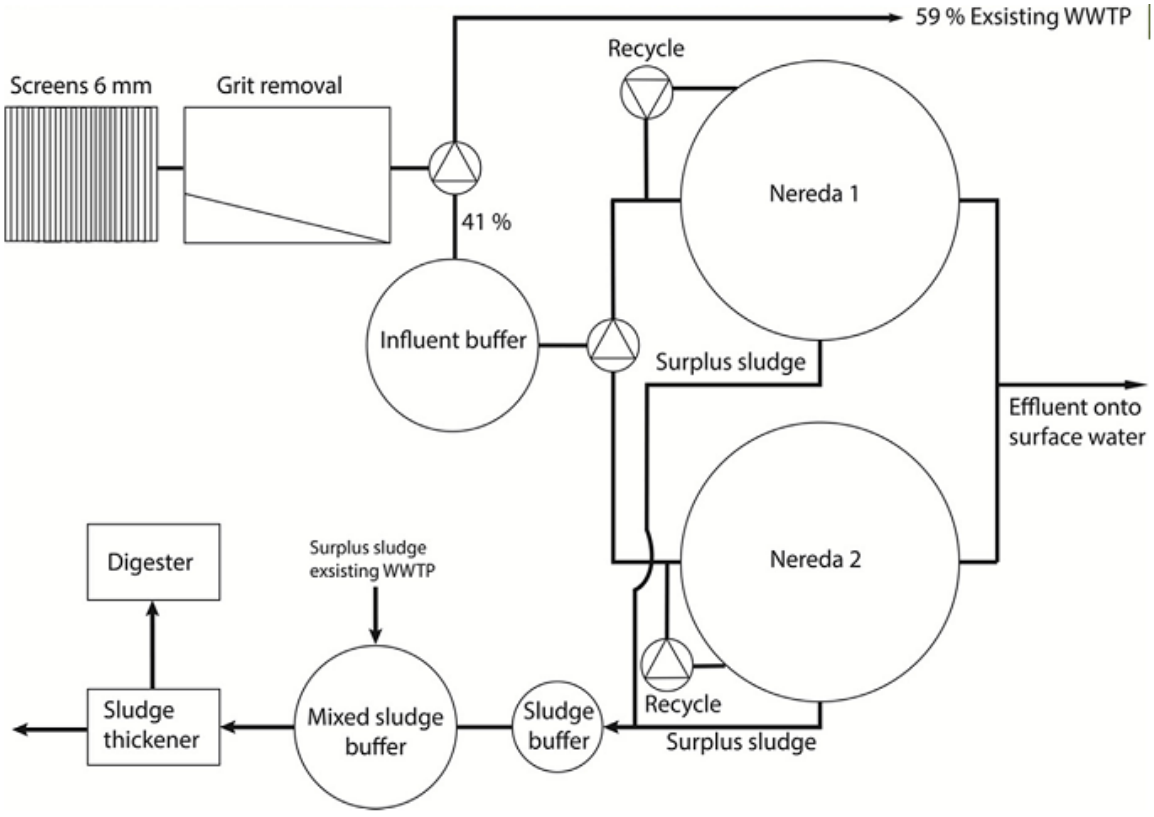


Figure 2-3: Process scheme of Garmerwolde WWTP (Pronk, et al., 2015)

In the following table, the performance data of the treatment plant for the year 2014 is presented.

Table 2-3: Performance data of Garmerwolde WWTP – 2014. Adapted from Pronk, et al. (2015)

Parameter	Average Influent (mg/L)	Average Effluent (mg/L)	Removal efficiency (%)
BOD_5	224	9.7	96
COD	506	64	87
NH_4-N dissolved	39.0	1.10	97
TN	49.4	6.9	86
TP	6.7	0.9	87
TSS	236	20	92

2.1.2. *Description of AGS technology*

AGS is a relatively novel wastewater treatment technology that has been in study for the last 20 years at laboratory-scale. Recently, full-scale systems have been installed around the world as SBR, with successful results for treating both domestic and industrial wastewater, and for upgrades or newly built plants.

AGS treatment technology resembles AS systems in the fact that, a mass of microorganisms (biomass; sludge) suspended in a liquid and developed in aerobic conditions, is responsible for purifying the influent wastewater of a reactor. In AS systems, the biomass forms flocs. In contrast, in AGS systems, agglutination of biomass in the form of compact granules, which have an extremely good settle-ability, is dominant and stimulated by design and control mechanisms (Giesen and Thompson, 2013). According to Adav, et al. (2008), settling velocities may reach 25 to 70 m/h, whilst for sludge flocs it might vary between 7 and 10 m/h. In the following figure, a picture comparing AGS with AS settle-abilities is shown.

Figure 2-4: Comparison of Nereda sludge and Activated Sludge after 5 minutes settling (Giesen and Thompson, 2013)

This excellent settle-ability of granules coupled with the sequential operation, implies that AGS systems do not require a secondary clarifier (De Bruin, et al., 2004) and therefore can work with high concentrations of biomass. Furthermore, no mixing equipment is required (De Bruin, 2013). Aerobic, anoxic, and anaerobic zones are present within the granules, thus, separate compartments apart from the main reactor are not required, and recycling pumps are minimized (Giesen and Thompson, 2013). As a result, AGS systems have a very small footprint, representing 25% of the conventional systems (De Bruin, et al., 2004), and lower energy consumption than conventional systems, presenting a minimum saving of 20% (De Bruin, 2013), which leads to lower construction and operating costs. However, it should be taken into account that according to De Bruin, et al. (2004), AGS WWTPs with high Rain Weather Flow (RWF) contribution would not be as competitive with AS as in the case of low RWF, because the maximum batch volume increases considerably with high RWF, increasing the volume of the AGS plant.

AGS process stability is remarkable and the effluent quality is excellent (De Bruin, 2013). At laboratory-scale research, removal efficiencies for COD, nitrogen, and phosphate resulted in 100% for the first one and 94% for the latter ones (De Kreuk, 2006). Removal efficiencies for full-scale systems can be seen in Table 2-1, Table 2-2, and Table 2-3.

In 1997 Morgenroth, et al. (1997) developed AGS in an SBR, and since then, SBR has been used for aerobic granulation due to the fact that Liu and Tay (2004) identified this type as a proper reactor configuration (Gao, et al., 2011). According to Castro-Barros (2013), the operational flexibility of SBR allows the development of proper granules since appropriate selection pressures, such as short settling time and high shear forces, can be applied.

The typical stages of AGS SBR operation are feeding, aeration, settling of biomass, and discharge (Winkler, 2012). A scheme of these is shown in Figure 2-5.

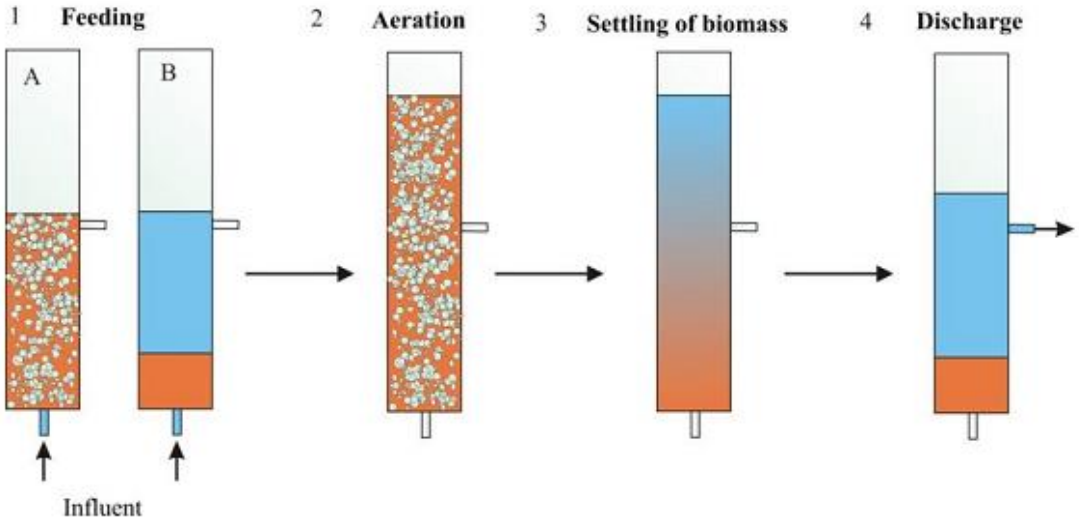


Figure 2-5: Scheme of cycle profile for AGS SBR. A: Mixed; B:Plug flow. (Winkler, 2012)

During the first AGS workshop held in Munich in 2004, researchers agreed on the definition of Aerobic granule, which is cited by De Kreuk, et al. (2007) as follows:

''Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs.''

According to De Kreuk, et al. (2007), in the same workshop it was also agreed that the minimum size of the granules should be 0.2 mm, size that would ensure the fast settlement of the particle. Giesen and Thompson (2013) point out another feature that defines aerobic granules; it is stated that SVI5 for AGS should be comparable to SVI30 for AS. Moreover Gao, et al. (2011) classify AGS as a ''type of self-immobilized microbial consortium''.

The process of granulation takes place in four different stages according to Adav, et al. (2008):

1. Contact between microorganisms to form aggregates by gravity, diffusion, thermodynamic, and/or hydrodynamic forces.
2. Initial attraction to form agglutination by physical, chemical, or biochemical forces.
3. Microbial forces like secretion of extracellular polymeric substances (EPS).
4. Hydrodynamic shear force that stabilizes the structure of the granule.

The most important conditions for developing proper granules are the following:

- Short settling time, which selects the biomass with good settle-ability, washing out with the effluent the biomass with slow settling velocities (De Bruin, et al., 2004).
- Conversion of readily biodegradable substrate into slowly biodegradable substrate by feast and famine regime (Pronk, et al., 2014). Anaerobic feeding periods select slow growing organisms, such as Polyphosphate Accumulating Organisms (PAO), which favour the formation of stable and dense granules (Winkler, 2012).
- High shear stress caused by intensive mixing favours the formation of smooth and dense granules (De Bruin, et al., 2004).

The formation of granules also depends on loading rates, substrate composition, reactor design, and seed sludge (Winkler, 2012).

Due to the Dissolved Oxygen (DO) gradient that exists within the granule, aerobic, anoxic, and anaerobic zones are present in layers, and therefore, organic matter and nutrients removal can take place simultaneously in the granule. This means that the microbial consortium that forms the granule is comprised of different layers of aerobic and facultative bacteria (Gao, et al., 2011), which include heterotrophs, nitrifiers, denitrifiers, PAO, and Glycogen Accumulating Organisms (GAO) (Adav, et al., 2008). Heterotrophs can perform organic degradation; whilst nitrifiers perform nitrification, converting ammonium to nitrite and nitrate; and denitrifiers perform denitrification, converting nitrate to nitrogen gas. Phosphorus removal might be performed biologically by PAOs and denitrifying PAOs, and/or physically by phosphorus precipitation of the aerobic granules (Gao, et al., 2011). Furthermore, consortia consisting of anaerobic ammonium-oxidizing (ANAMMOX) bacteria have also been developed (Gao, et al., 2011). Granules present the same functional groups of bacteria as sludge flocs, but they are quite different in shape and structure (Winkler, et al., 2013), as shown in Figure 2-6.

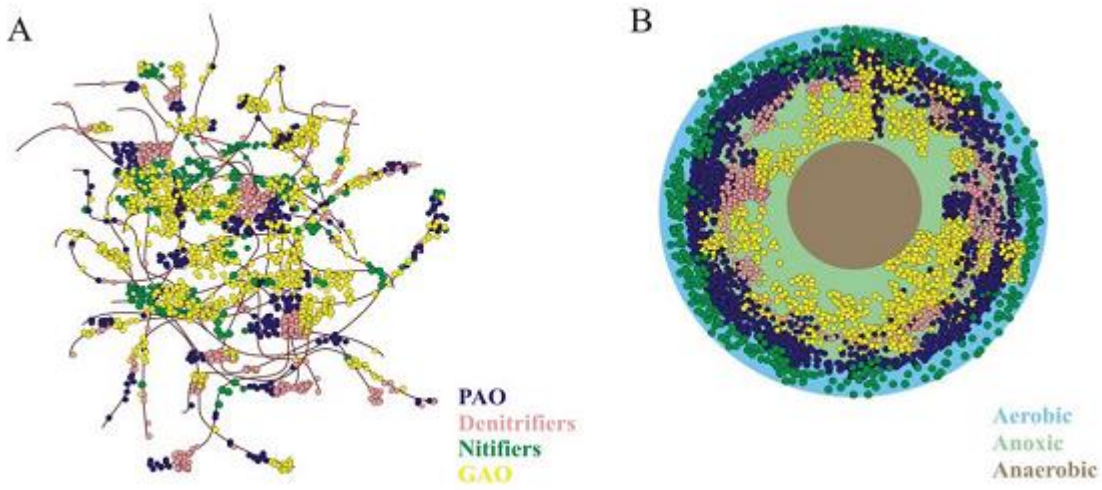


Figure 2-6: Scheme of microbial communities in a sludge floc (A) and in a granule (B) (Winkler, 2012)

Furthermore, according to Winkler, et al. (2013), due to shear stress, substrate gradients, and the presence of protozoa in the outer layers, aerobic granules provide many ecological niches. They also point out that segregation of biomass within the height of the reactor occurs; the top communities differ from the bottom ones. According to them, comparing changes in AS microbial populations during the time with those in AGS, the first ones stayed more or less as the initial population, whilst granule communities changed all the time. They also state that both AGS and AS communities growth rate might be altered by fluctuations in the influent, such as nutrients availability and temperature.

According to Gao, et al. (2011), the research efforts until that moment had been focused on the factors influencing granulation, cultivation conditions, and the microbial community that conforms the granular sludge. As mentioned before, there are no studies reported about removal of pathogens in AGS.

2.2. Pathogens in wastewater

According to Henze, et al. (2008), microorganisms which cause disease and are transmitted by faecal-oral route are defined as enteric pathogens. They also state that domestic wastewater presents a plethora of these microbes originated from faeces of infected people. Bacteria, viruses, protozoa, and helminths are pathogens commonly found in raw wastewater (Henze, et al., 2008); in the following table, some relevant enteric pathogens are indicated, giving also the average concentration of each in raw domestic wastewater.

*Table 2-4 Pathogens in raw domestic wastewater. Adapted from Metcalf (2003)/ *(Monis, 2015)*

Group	Pathogen	Concentration (MPN/100 mL)
Bacteria	*Salmonella*	10^2 - 10^4
	Pathogenic *Escherichia coli*	Low*
Protozoa	*Cryptosporidium parvum* oocysts	10^1 – 10^3
Virus	Enteric virus	10^3 - 10^4

Although these are common values, according to Henze, et al. (2008), concentration of pathogens in raw wastewater might vary a lot between different geographical regions depending on the current amount of people infected, socio-economical characteristics, and per-capita water consumption. The more people infected, the lower socio-economical level, and the lower per-capita water consumption, the higher the concentration of pathogens in domestic wastewater. All of these characteristics are associated with developing countries, and therefore it can be stated that in developing countries, wastewater has a higher concentration of pathogens than in developed countries (Henze, et al., 2008). At the same time, this increases the risk of human infection with pathogens in developing countries.

A short description of the pathogens indicated in Table 2-4 is presented below.

2.2.1. *Salmonella*

According to Henze, et al. (2008), *Salmonella* are the pathogenic bacteria most frequently studied in wastewater. They state that it is a vast group of more than 2,400 pathogenic serotypes which are gram-negative, rod-shaped, and might provoke a wide range of diseases, from mild gastroenteritis to severe illness or death. They mention that *Salmonella typhi* and *Salmonella paratyphoid* are usually found only in humans, causing typhoid and paratyphoid fever respectively. *Salmonella* spp. can usually be found in wastewater because according to Madigan, et al. (1997), it colonizes the intestine of both cold- and warm-blooded animals. In Figure 2-7, a Gram-stain of *Salmonella* cells is shown.

2.2.2. *Escherichia coli*

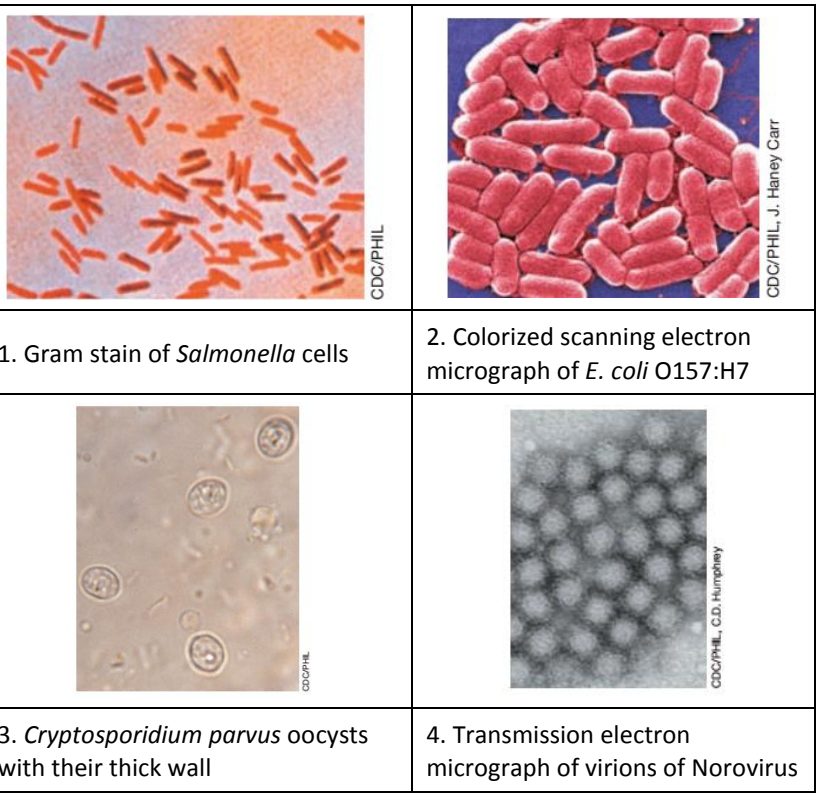
According to Henze, et al. (2008), *Escherichia coli* is a bacteria commonly found in the gastrointestinal tract of all warm-blooded animals, it is gram-negative, rod-shaped, and usually non-pathogenic. Nevertheless, they state that there are strains, such as enterohemorrhagic *E. coli* (EHEC), which are potentially pathogenic and might provoke gastroenteritis with severe diarrhoeal disease and urinary tract distress. *E. coli* O157:H7 is the most common EHEC and it is usually found in the intestines of healthy cattle (Madigan, et al., 1997). Both humans and cattle can emit EHEC (Henze, et al., 2008) in their faeces, and therefore will be present in wastewater. In Figure 2-7, a colorized scanning electron micrograph of *E. coli* O157:H7 is depicted.

2.2.3. *Cryptosporidium*

Cryptosporidium is one of the most relevant protozoa associated with several waterborne disease outbreaks in developed countries (Henze, et al., 2008). According to them, this organism has a very resistant stage called oocyst, which can survive chlorine disinfection, but, as it is much bigger than bacteria, it can be removed in large amounts by granular media filtration. As they point out, only a small amount of oocysts is needed to infect human; both *C. parvum* and *C. hominis* are species which infect human, and they may also infect cattle. They also state that *Cryptosporidium* oocysts are not excreted by humans as much as other protozoa, like *Giardia* cysts, and therefore they are found in a lower quantity in wastewater. In Figure 2-7, *C. parvum* oocysts with their thick wall are shown.

2.2.4. *Enteric viruses*

According to Henze, et al. (2008), enteric viruses are those viruses which inhabit the gastrointestinal tract of human beings. They also point out that as they are known for being very host-specific, human viruses can only be transmitted to human beings; as in the case of *Cryptosporidium*, only a small amount of viruses is needed for infection. Known enteric viruses, which at present are more than 160, usually cause diarrhoea. According to them, some enteric viruses are Enteroviruses, Rotavirus, Adenovirus, and Norovirus. In Figure 2-7, a transmission electron micrograph of virions of Norovirus is shown.



1. Gram stain of *Salmonella* cells	2. Colorized scanning electron micrograph of *E. coli* O157:H7
3. *Cryptosporidium parvus* oocysts with their thick wall	4. Transmission electron micrograph of virions of Norovirus

Figure 2-7: Images of pathogens present in wastewater (Madigan, et al., 1997)

2.3. Indicator organisms

According to Henze, et al. (2008), the examination of pathogens in water is an expensive, time-consuming, and tough task, which can be avoided with the use of indicator organisms. They state that these are non-pathogenic organisms which are present in the excreta of all warm-blooded animals. Indicators can be grouped by their intended purpose as follows.

Table 2-5: Groups of indicators. Adapted from Henze, et al. (2008)

Group name	Definition
Process indicator	A group of organisms that demonstrate the efficacy of a process.
Faecal indicator	A group of organisms that indicate the presence of faecal contamination.
Index and model organism	A group or species indicative of pathogen presence and behaviour respectively.

According to Henze, et al. (2008), examples of index organisms are *Escherichia coli* for *Salmonella* and F-specific coliphages for human enteric viruses.

2.3.1. *Escherichia coli as indicator*

According to Henze, et al. (2008), *Escherichia coli* is part of the group Faecal Coliforms, which is part of a larger group called Total Coliforms. They point out that Total Coliforms are comprised of all aerobic and facultative anaerobic, non-spore forming, gram-negative, rod-shaped bacteria, which ferment lactose with gas production in prescribed culture media within 48 hours at 35°C; whilst Faecal Coliforms can also produce acid and gas by fermenting lactose within 24 hours at 44.5°C. Despite Total and Faecal Coliforms have been used as indicators for several years, recently, their correlation with faecal contamination is in doubt, and therefore *E. coli* is being used more frequently, which is easy to recognize within the Faecal Coliform group. The concentration of Faecal Coliforms in raw wastewater ranges between 10^6 and 10^7 CFU/100 mL (Henze, et al., 2008). According to Madigan, et al. (1997), *E. coli* is part of the phylum Proteobacteria, and it is classified as Gamma-proteobacteria.

2.3.2. *Bacteriophage as indicator*

Due to the fact that bacteriophages (bacterial viruses) resemble viruses regarding structure, morphology, size, and behaviour in aquatic environments, they have been used as viral pollution indicators in various applications, such as evaluation of virus resistance to disinfectants, surface and groundwater tracers, and fate of viruses in water and wastewater treatment (Henze, et al., 2008). According to them, the most studied groups of bacteriophages are the following:

- Somatic coliphage, which infects *E. coli* strains through cell wall receptors.
- F-specific RNA coliphage (male-specific phage), which infects *E. coli* strains and related bacteria by F+ or sex pili. MS2 and ΦX174 are examples of this coliphage.
- *Bacteroides Fragilis* phage, which is not frequently used as a routine indicator because the host is an anaerobic bacteria, and this implies complexity in the analytical procedures.

Concentrations of F-RNA coliphage in raw wastewater are between 10^3 and 10^5 PFU/100 mL (Monis, 2015).

2.4. Removal and fate of pathogens in AS

Regarding pathogen removal in AS systems, there are some studies which investigated the removal efficiencies and the fate of the pathogens. As AGS and AS are similar technologies in some aspects, one could say that the removal processes would be comparable. However, the fact that flocculent microorganisms behave very different than those agglomerated in granules does not permit to make a prediction about this topic. Indeed, De Kreuk (2006) state that, due to the differences between these two groups of microbes regarding their density and their interaction with suspended solids, AGS pathogen removal capacity is expected to be different from that of AS. Nevertheless, the removal of pathogens in AS systems is relevant for this research because it allows to set a reference to which compare the novel AGS technology, thus, a summary of the literature found in this regard is presented in the following paragraphs.

According to Henze, et al. (2008), pathogen removal in AS systems varies a lot depending on the retention times of the system. Nonetheless, they state that adsorption onto the biological flocs and post-sedimentation, virus inactivation by bacteria, and ingestion by protozoa and small nematodes, are the most common mechanisms of pathogen removal. Bitton (2005) states that in AS systems, bacteria are removed by inactivation, predation by ciliated protozoa (especially for free-suspended bacteria), and adsorption to solids or enmeshment in the flocs, or both, with post-sedimentation.

As mentioned before, in some cases, pathogens get attached to sludge flocs, and therefore, if there is poor settling and high concentrations of suspended solids end up in the clarified effluent, probably high concentration of pathogens are also being discharged with the effluent (Chahal, et al., 2016).

Henze, et al. (2008) also point out that the removal efficiencies in AS for protozoa and enteric viruses vary within a wide range, from 4 to 93% and from 0 to 98% respectively; whilst the removal of coliphage correspond to 90 to 99%. Bitton (2005) states that the removal of indicator and pathogenic bacteria is reported to be between 80 and 99%. Most of the viruses and bacteria are transferred to the sludge (Henze, et al., 2008).

Wen, et al. (2009) performed a study of the fate of pathogens and indicators in an AS laboratory WWT system fed with wastewater from an AS WWTP. The results showed log removal efficiencies of 2.06 (±0.26) for pathogenic *E. coli*, 2.37 (±0.41) for Total Coliforms, 1.85 (±0.25) for MS2 bacteriophage, 2.41 (±0.16) for *Cryptosporidium*, and 2.49 (±0.15) for *Giardia*, according to them, these results are in accordance with previous works.

Regarding coliphage, Wen, et al. (2009) concluded that the major removal mechanism of MS2 in their experiment was adsorption onto the bacterial floc. Furthermore, they state that the primary method for bacterial removal is predation by protozoa, and for protozoa removal is attachment to bacterial floc and subsequent sedimentation.

Van der Drift, et al. (1977) studied the removal of *E. coli* in wastewater in AS. They concluded that the removal of *E. coli* in AS is a biphasic process, first a rapid removal due to attachment to sludge flocs predominates following a Langmuir adsorption isotherm, and later there is a slower but more significant elimination due to predation by ciliated protozoa. They state that predation by ciliates is the main mechanism of *E. coli* removal in AS.

2.5. Protozoa in AS

Protozoa play a major role in the AS process (Pauli, et al., 2001). They are unicellular, heterotrophs (Bitton, 2005), eukaryotic organisms (Madigan, et al., 1997). According to Bitton (2005), their dissimilation is either by the absorption of dissolved nutrients or by the ingestion of particulate matter, such as bacteria. According to Pauli, et al. (2001), only few specialists survive under strict anaerobic conditions, but there are some facultative anaerobes. Division of protozoa is often by binary fission (Bitton, 2005).

According to McKinney and Gram (1956), there are three classes of protozoa of importance to the AS process: *Sarcodina, Mastigophora, and Ciliata* (or ciliates). The dominant class in AS systems are ciliates, which improve the treatment due to feeding on bacteria (Pauli, et al., 2001). Ciliates use mainly organic particulate matter as food, they move by means of cilia, and they are subdivided into free-swimming ciliates and stalked or sessile ciliates; an example of this last subdivision is the genus *Vorticella* (McKinney and Gram, 1956), which is part of the Peritrichia subclass. Pauli, et al. (2001) also included the crawling ciliates as a subdivision, and they state that stalked and crawling ciliates can bound to bacterial agglomerations, such as flocs.

According to Pauli, et al. (2001), ciliates can uptake nutrients by different mechanisms:

- Substances can reach the cell through its plasma membrane.
- Pinocytosis, which consists on transferring soluble substances in vacuoles from the plasma membrane to the interior of the cell.
- Phagocytosis, which consists of particulate matter uptake. The oral apparatus of ciliates is particularly specialized for this type of feeding.

According to them, the phagocytosis of the ciliates, or so-called filter-feeding or grazing, starts with the generation of a water current by the cilia, which concentrates the particulate matter present in a great amount of liquid by a filtration process, retaining particles in the size between 0.3 and 5 µm. They state that the process continues with the intake of the retained particulate matter in vacuoles. According to them, *Vorticella microstoma*, which average size is 60 x 30 µm, can achieve a filtration rate of 156 nL/h when there are bacterial densities of 10^6 individuals/mL (ind/mL henceforth). The following picture illustrates this process.

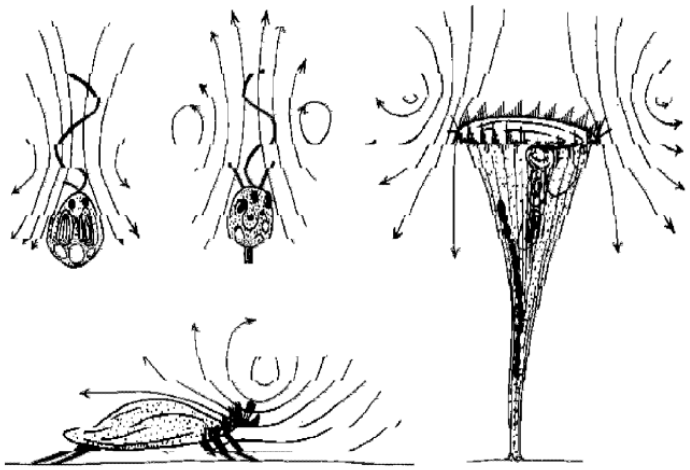


Figure 2-8: Filter-feeding mechanism of ciliates (Pauli, et al., 2001)

The ciliate community in AS systems is majorly comprised of filter-feeding organisms (Pauli, et al., 2001). If bacteria find places to stay out of reach of their predators, like flocs, the growth rate of ciliates will be diminished (McKinney and Gram, 1956). In the same line, Pauli, et al. (2001) state that the ability of bacteria to grow collectively in larger forms, such as flocs and biofilms, protects them from being eaten by ciliates which are able to eat or filter particles only within certain size ranges.

McKinney and Gram (1956) state that stalked ciliates have less energy requirements than free-swimming ciliates, therefore, if there are less free-suspended bacteria available than the number needed by free-swimming ciliates, the stalked ones will predominate. Thus, according to them, a low number of stalked ciliates and no other protozoa present in AS systems, indicates a predominance of flocs compared to free-suspended or dispersed bacteria, i.e. it indicates a stable system. The next figure depicts the variation of the amount of microorganisms with the time in an AS system, and which microorganisms predominate when there are more bacteria dispersed or flocculated.

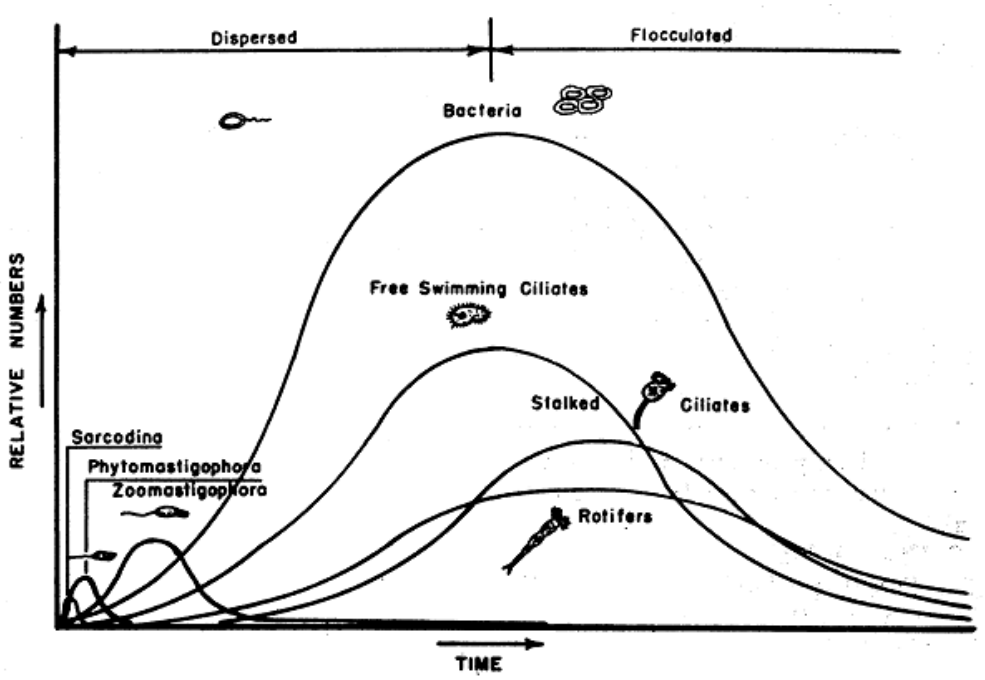


Figure 2-9: Microorganisms' predominance in AS (McKinney and Gram, 1956)

According to Pauli, et al. (2001), doubling times for *Vorticella microstoma* and *Vorticella convallaria* in municipal AS plants are 5.0 and 7.6 hours respectively, and a typical population density of the first one is 0.59 x 10^4 ind/mL.

2.6. Protozoa in AGS

Recent studies confirm that stalked ciliates attach to the granules of AGS lab and full-scale systems (De Kreuk, et al., 2010, Lemaire, et al., 2008, Li, et al., 2013, Pronk, et al., 2015).

Lemaire, et al. (2008) found a myriad of stalked ciliates of the subclass Peritrichia attached to the granules of a lab-scale AGS reactor fed with abattoir wastewater. They state that clusters of ciliates were present in almost every analysed granule, and due to the high shear force applied, they were placed in sheltered areas, such as concave parts of the granules or interstices.

Weber, et al. (2007) explain that stalked ciliates of the subclass Peritrichia play a major role in the granulation process of an AGS system, serving as substratum for the bacterial community growth and granule development. This can be observed in Figure 2-10, which was obtained with SEM and shows bacteria attached to stalked ciliates before the granulation process starts, using the ciliate as the substratum. They also state that ciliates might play a role in the formation of the EPS involved in the granulation. Furthermore, they observed in their experimental set-ups (three different reactors fed with malt house wastewater, brewery wastewater, and synthetic wastewater) that when the granules were already formed and matured, stalked ciliates were always part of their fringe zone. They detected large quantities of bacteria inside ciliates food vacuoles using FISH and SEM. Figure 2-11 shows a mature

granule from an AGS lab-reactor fed with synthetic wastewater with stalked ciliates from the subclass Peritrichia attached in a picture obtained with SEM (Weber, et al., 2007).

According to De Kreuk, et al. (2010), protozoa presence in AGS systems lead to less effluent suspended solids. Furthermore, Li, et al. (2013) quantified this reduction when studying the dwelling of *Vorticella* by granules in an AGS lab-scale reactor fed with domestic wastewater; the results showed that when protozoa were absent, the average TSS in the effluent was 103 mg/L, whereas when *Vorticella* were present, a value of 84.7 mg/L was obtained on average, with a minimum of 47 mg/L for a *Vorticella* population density of 2.4 x 10^4 ind/mL. On the other hand, they found out that the presence of these ciliates negatively affected the settling velocity and the SVI of the granules.

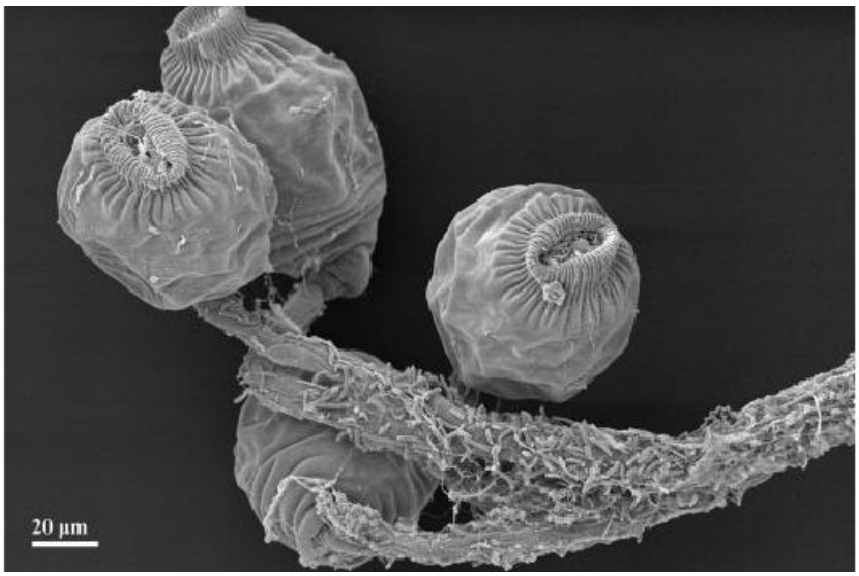


Figure 2-10: Bacteria attached to stalked ciliates (Weber, et al., 2007)

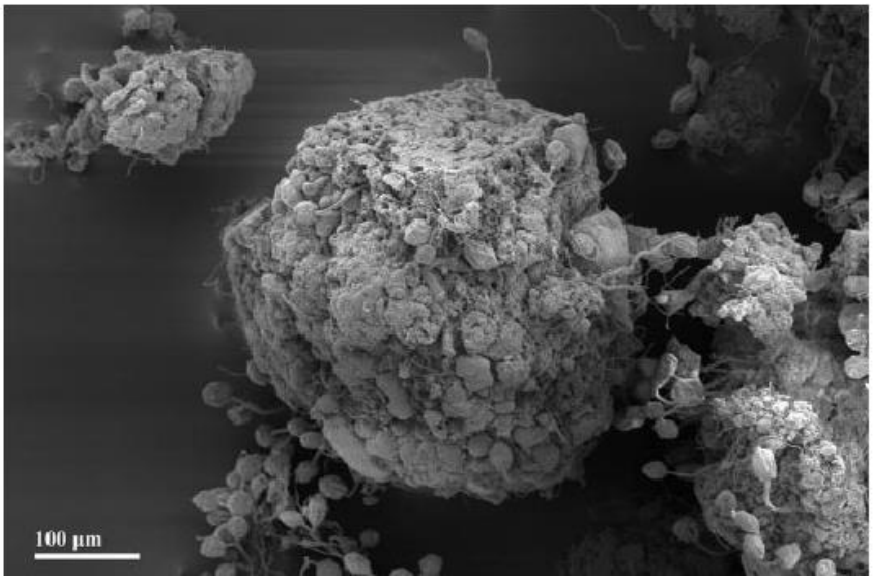


Figure 2-11: Stalked ciliates attached to mature granules (Weber, et al., 2007)

2.7. Association pathogens-particles in conventional wastewater treatment

According to Chahal, et al. (2016), association between pathogens and particles play a major role in determining the fate of pathogens in wastewater treatment, and therefore the characteristics of this association as well as the influencing factors for its formation and stability are relevant for this study. In the following paragraphs, a description of the characteristics of the association pathogen-particles for conventional wastewater treatment, such as AS, will be presented.

Sludge flocs in conventional wastewater treatment are comprised of a mixture of fine, colloidal, and large particles, which include a wide range of microorganisms, as well as non-living material, such as EPS (Chahal, et al., 2016). According to them, bacteria, viruses, and protozoa present in wastewater can get attached to these sludge flocs; the typical structure of a bacterial floc is depicted in the following figure.

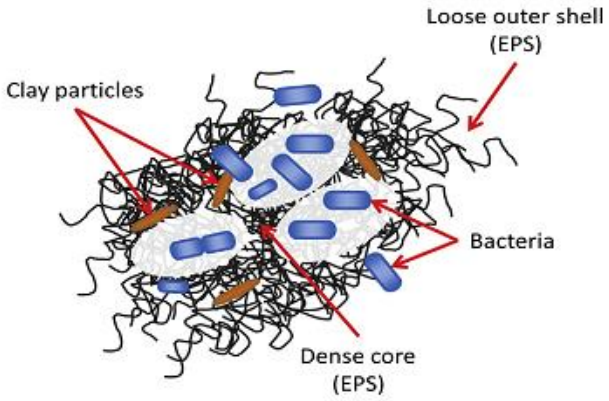


Figure 2-12: Typical structure of a bacterial floc (Chahal, et al., 2016)

According to Chahal, et al. (2016), the attachment of microorganisms to sludge flocs might be provoked by electrostatic attractions, physical entrapment, or hydrophobic interactions. They also state that with time, disaggregation takes place, and also new associations are formed. As pointed out by them, pathogens associated with dense particles will settle rapidly, whilst microbes attached to lighter particles will survive longer as they will tend to remain suspended in the liquid. Figure 2-13 shows a scanning electron microscope image of *Escherichia coli* attached to different wastewater particles.

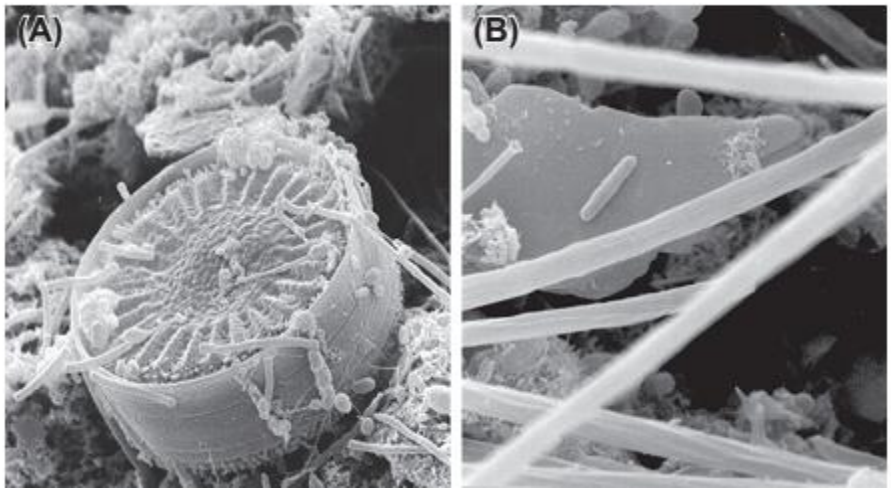


Figure 2-13: Escherichia coli attached to diatom in a biofilm (A) and to clay particle (B) (Chahal, et al., 2016)

2.7.1. *Association bacteria-particle*

There are different mechanisms of bacteria-particle association in conventional wastewater treatment, such as adsorption of the bacteria onto the particle surface, bacteria harbouring in particle cracks, or adhesion of bacteria to biofilms. This association depends on many factors, such as particle composition, particle size, and the age of the bacteria; examples of these are the cases in which a bacterium is attracted and colonizes a particle for its affinity with the nutrients released by the particle, or the adsorption of bacteria onto the surface of inorganic particles (Chahal, et al., 2016).

Derjaguin, Landau, Verwey, and Overbeck (DLVO) double layer theory explains the adsorption of bacteria onto particle surface stating that the attraction of bacteria takes place in two steps: first, weak Van der Waals forces and repulsive electrostatic forces are reversibly overcome by the cell with physical forces (changes in medium's ionic composition or hydraulic shear forces) within 5 and 10 nm from the particle surface (secondary energy zone); after this, adhesion may occur, which implies the formation of a permanent union between bacteria and particle surface involving a lot of energy, within 1 nm from the particle surface (primary energy zone) (Chahal, et al., 2016). According to them, EPS might play a role in the adhesion step by forming dipole-dipole interactions or hydrogen bonds.

According to Chahal, et al. (2016), as biofilms need permanently growing bacteria, and pathogenic bacteria are unlikely to reproduce themselves under the typical temperature and nutrients availability of wastewater treatment systems, heterotrophic bacteria will probably form the biofilms, which acts as a substrate for attachment of pathogenic bacteria, viruses, and protozoa.

Specific studies carried for coliform bacteria demonstrated that these bacteria, which have a size between 1 and 10 µm, were associated with particles with diameter greater than 10 µm (Chahal, et al., 2016). They state that it was proved that in AS, the association coliform-particle decreased with the increase of Sludge Retention Time (SRT), however, it could not be demonstrated if SRT affected the binding, or if it was just a matter of natural die-off with time.

2.7.2. *Association virus-particle*

According to Chahal, et al. (2016) association between virus and particles had not been yet studied sufficiently. Nevertheless, they state that enteric viruses are much smaller than bacteria, 15 to 80 nm and 1 to 10 µm respectively, and that these viruses are commonly associated with particles with diameter smaller than 10 µm, such as sand, clay, suspended colloids, or EPS. Also, they mention that the virus-particle association is by adsorption of the virus onto particle surface by hydrophobic and electrostatic interactions.

2.7.3. *Association protozoa-particle*

Association between oocysts and cysts of protozoa, such as *Cryptosporidium* and *Giardia*, and suspended wastewater particles had not been yet studied sufficiently, however, it is expected that some factors which determine bacteria-particle associations, may also have an influence on this association (Chahal, et al., 2016). According to them, *Cryptosporidium* oocysts and *Giardia* cysts might have different mechanisms of attachment to particles, since the first ones are negatively charged and the latter ones are hydrophobic. They also state that these pathogens can interact with biofilms, influencing the removal of them in wastewater treatment.

2.8. Current situation of domestic wastewater management in Uruguay

The current situation of domestic wastewater management in Uruguay has been improved in the last years with the construction and development of projects of new wastewater treatment plants, however, some of these projects are delayed due to lack of funding. Moreover, the capital city Montevideo still relies only on a wastewater pre-treatment plant with a 2 km subaquatic discharge pipe to the estuary Río de la Plata. This means that some localities in Uruguay need alternative solutions for their planned and current wastewater treatment systems, which should be more cost-effective than conventional treatments, and at the same time, ensure good effluent quality. Therefore, AGS treatment technology might be a potential solution for this problem; to evaluate this, an analysis of the applicability of AGS to Uruguay is proposed to be done as part of this Master's thesis. In this section, a brief description of the current sanitation situation in Uruguay is presented.

Uruguay is a country located in the Atlantic Coast of South America between Brazil and Argentina. Its population was projected to be 3,487,500 in 2016 (Instituto Nacional de Estadística [National Institute of Statistics], 2013), and the total surface is around 176,000 km^2. With only 194 km^2, the capital city Montevideo, is home for almost half of the population of the country. The rest of the population is spread in the other 18 departments of the country in rural areas and cities.

The sanitation situation in Uruguay has been recently receiving public interest since some events of eutrophication in the main sources of drinking water of the country had affected the drinking water quality supplied by the utility OSE to the community, twice in the last five years. After these events, the Ministry of Housing, Land Use, and Environment (MVOTMA) decided

to apply a plan to protect the river basin Santa Lucía which supplies drinking water to the capital city and its metropolitan area.

The management of domestic wastewater is responsibility of the municipality in the case of Montevideo, and of OSE in the rest of the country. The following figure depicts the sanitation situation of the capital cities of Uruguay based on OSE (2016a). As can be seen, all capital cities of the departments have sewage systems, however, there are 4 out of 19 which have direct discharge of the sewage into water bodies, and two which only have a pre-treatment plant. Taking a look at the population of each capital city, it can be observed that the most populated, which is Montevideo, has only pre-treatment, whilst the two that follow, Salto and Paysandú, have direct discharge.

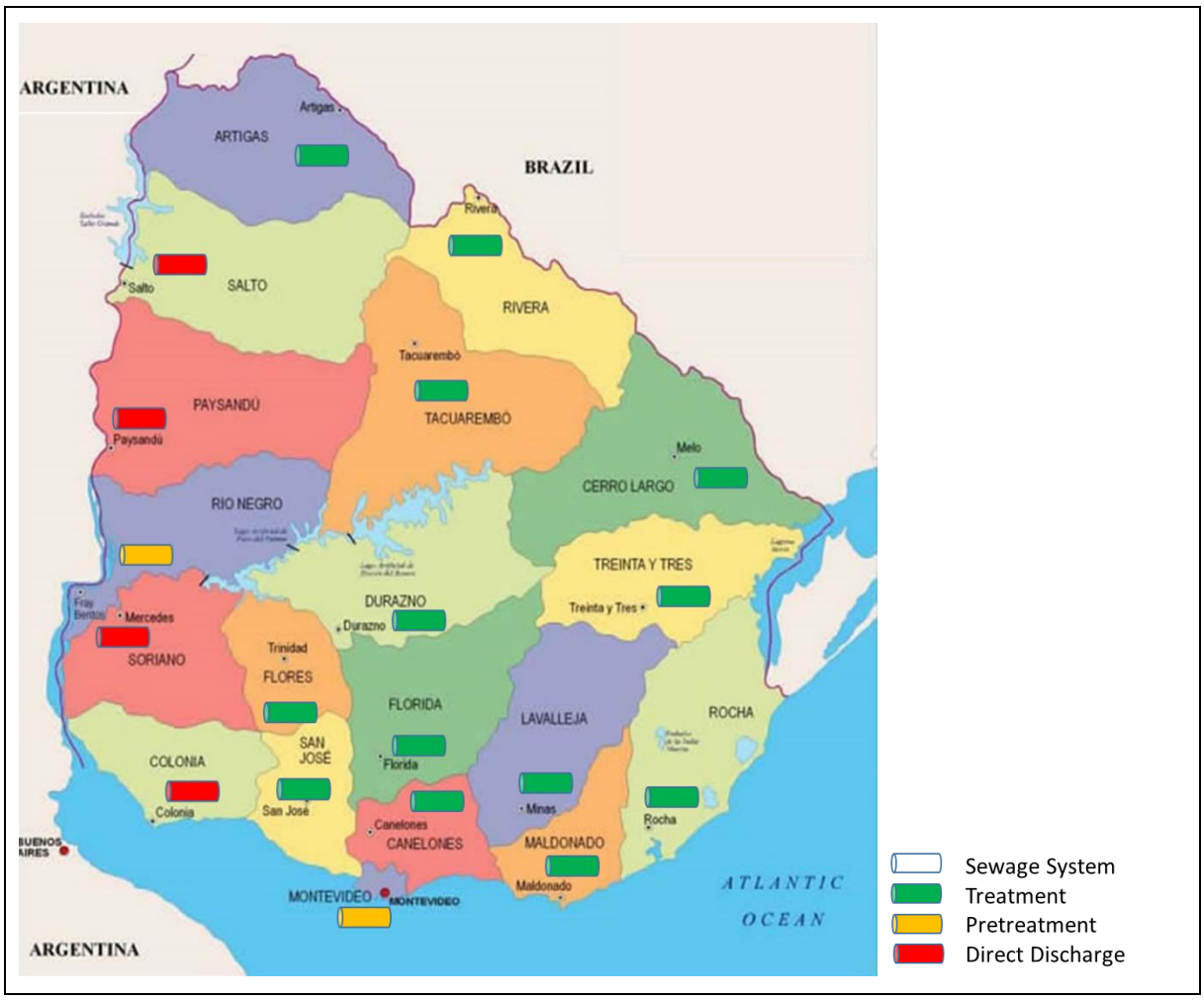


Figure 2-14: Sanitation situation in capital cities of Uruguay. Based on data from OSE (2016a).

Uruguayan standards for municipal and industrial effluent discharges were established in 1973 by Decree 253/79 (MVOTMA, 1973), and they have been regulated by the National Environmental Authority DINAMA, which is part of the MVOTMA. In the following table, the current standards for discharge into a water course for relevant parameters for this research are presented.

Table 2-6: Uruguayan standards for discharge into a water course. Adapted from MVOTMA (1973)

Parameter	Value
pH	6.0 to 9.0
BOD	60 mg/L
COD	No standard
Ammonia	5 mg N/L
TP	5 mg P/L
TSS	150 mg/L
Fats and oil	50 mg/L
Faecal coliforms	5,000 CFU/100 mL

Despite these have been the standards since 1973, as a general practice, DINAMA was not fining companies or entities for ammonia or TP incompliances. Given the occurrence of the events explained before, and as part of the strategy to protect Santa Lucía's basin, in 2013 the MVOTMA intimated OSE and some industries which were discharging their effluents in this basin, to comply concentrations of 10 mg N/L for TKN, 10 mg N/L for nitrate, and all the standards established by Decree 253/79 and Modifications (MVOTMA, 2013a, MVOTMA, 2013b).

In the following points, the sanitation situation of two different cases will be addressed more in detail. The first case is Montevideo, which as the capital city and most populated and dense locality in Uruguay, only relies on a domestic wastewater pre-treatment plant. The second case is Mercedes, the capital city of the department of Soriano, which currently has direct discharge of its domestic wastewater into the river Río Negro. This river is a compromised water course regarding eutrophication events, and presence of faecal coliforms downstream Mercedes city (DINAMA, 2011). A project for a domestic wastewater treatment plant for Mercedes has been developed, but its execution is delayed due to lack of funding (OSE, 2016b).

2.8.1. *Sanitation situation in Montevideo*

As mentioned before, Montevideo is the capital city of Uruguay. Its population was projected on around 1,375,000 people for the year 2016 (Instituto Nacional de Estadística [National Institute of Statistics], 2013). Its average temperature is 16.7°C and its annual rainfall is 1,101 mm (Instituto Uruguayo de Meteorología [Uruguayan institute of meteorology], 1961-1990). Montevideo had the first sewage system in South America, dated back to the 1850s (Intendencia de Montevideo [Municipality of Montevideo], 2015). At present, there are

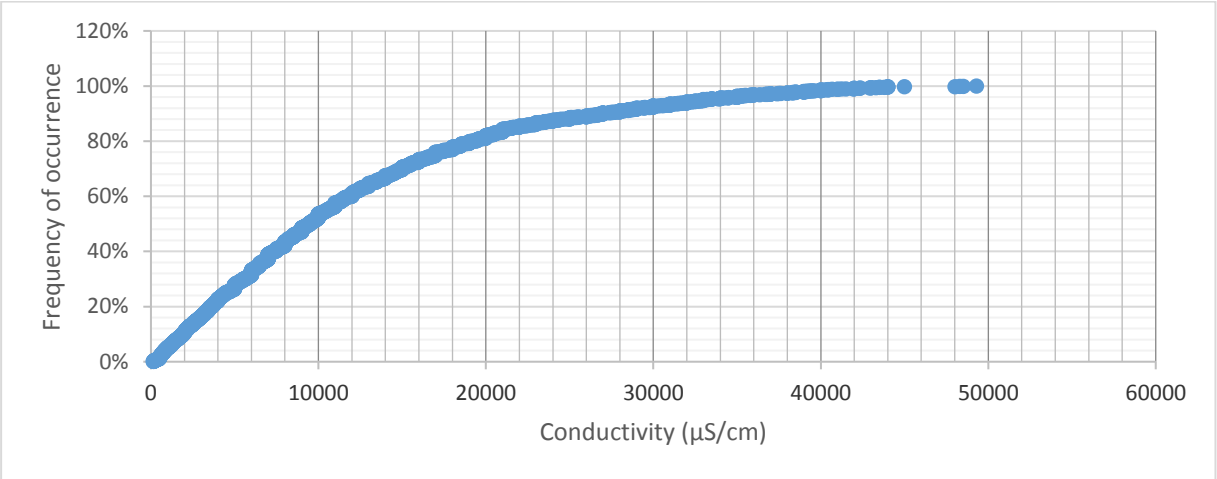
different subsystems within the sewage network, but the main subsystem is the one that ends in Punta Carretas' wastewater pre-treatment plant, located in the southernmost point of Montevideo. This plant comprises screens, grit and fat removal chambers, and a subaquatic final pipe of 2 km long which discharges in the estuary Río de la Plata.

In the following tables, the average concentration of different parameters of the pre-treated discharged wastewater is presented. The data was provided only for academic purposes by the Service of Quality Evaluation and Environmental Control of the Department of Environmental Development from the Municipality of Montevideo, and it was measured between the years 2012 and 2015, with approximately 10 samples per year.

Table 2-7: Montevideo's pre-treated wastewater characterization (Intendencia de Montevideo [Municipality of Montevideo], 2016b)

Parameter	Value
pH	7.5
BOD	106 mg/L
COD	250 mg/L
Ammonium	26 mg N/L
TP	3.8 mg P/L
TSS	103 mg/L
Fats and oil	< 25 mg/L
Total coliforms	5,5 x 10^6 CFU/100 mL

Conductivity is not measured in the plant, but it is measured in the Combined Sewer Overflows (CSO) of the network. In the following graph, the frequency of occurrence of the conductivity values of the wastewater between the years 2010 and mid-2016 are shown; these data was also provided by the Service of Quality Evaluation and Environmental Control of the Municipality of Montevideo.



Graph 2-1: Frequency of occurrence of conductivity – Punta Carretas' subsystem CSO's

From the previous graph, it can be observed that in 80% of the measurements, the conductivity was above 3,000 µS/cm. Furthermore, the average conductivity is 12,000 µS/cm, which is extremely high taking into account that according to Water Environmental Federation (1998), domestic wastewater conductivity usually ranges between 50 and 1,500 µS/cm. This can be due to the fact that the main sewers are located parallel to the estuary Río de la Plata, which might be a source of saline intrusion into the sewer. Another source of high conductivity may be the discharge of industrial effluents into the system. The following table shows the industrial sectors which discharge wastewater in Punta Carretas' sewage system, indicating the number of industries for each sector (from higher to lower quantity).

Table 2-8: Industrial sectors in Punta Carretas' system (Intendencia de Montevideo [Municipality of Montevideo], 2016a)

Industrial sector	Number of industries
Food processing	13
Industrial and vehicle wash.	8
Tannery	7
Metallurgic and chemical.	5
Beverage production, pork processing, and fish processing.	4
Metal coating and printing.	3
Hospital laundry, textile, machinery maintenance, and dye production.	2
Laundry, fish oil production, oil and fat production, pharmaceutical, paper mill, leachate treatment, and paint production.	1

2.8.2. *Sanitation situation in Mercedes*

Mercedes is the capital city of the department of Soriano, with a population of 41,975 (Instituto Nacional de Estadística [National Institute of Statistics], 2011). Its average temperature is 17.3°C and its annual rainfall is 1,130 mm (Instituto Uruguayo de Meteorología [Uruguayan institute of meteorology], 1961-1990). As it can be observed in Figure 2-14, Mercedes has a sewage system that directly discharges into a water course, the river Río Negro. This is a river very much compromised in terms of occurrence of eutrophication events, high concentration of faecal coliforms downstream Mercedes city, and presence of high levels of heavy metals in some areas (DINAMA, 2011). This might be due to domestic, industrial, and agricultural effluent discharges. According to OSE (2016c), a tertiary wastewater treatment plant with disinfection is planned to be installed in Mercedes in the following 5 years, but no date is yet defined due to lack of funding. In the following table, the average concentration of a set of parameters of Mercedes' raw wastewater for the year 2015, with one sample per month, is presented. There is no official record of the industries that discharge into the sewage system.

Table 2-9: Mercedes' raw wastewater characterization (OSE, 2016c)

Parameter	Value
pH	7.7
BOD	238 mg/L
COD	490 mg/L
TKN	44 mg N/L
Nitrate	< 1.0 mg N/L
TP	4.5 mg P/L
TSS	142 mg/L
Fats and oil	67 mg/L
Escherichia coli	6.6×10^6 MPN/100 mL

2.9. Effects of salt concentration on AGS

Generally, a high concentration of salt in wastewater is associated with industrial discharges, however, domestic wastewater might also contain a high salinity; such is the case of coastal areas with high seawater intrusion into sewers or seawater toilet flushing practices (Pronk, et al., 2014). According to Bassin, et al. (2011), high salt concentrations in influent wastewater negatively affect organic matter, phosphorus, and nitrogen removal in biological wastewater treatment plants.

Pronk, et al. (2014) conducted an experiment in an AGS SBR fed with synthetic wastewater containing different salt concentrations, and they found out that although the granules were stable, their size decreased and the effluent turbidity increased significantly for the highest salinity tested, which was 20 g/L Cl^-. They attributed the decrease in granule size to an adverse effect of high salinity on EPS; they state that EPS might be weakened by the replacement of the calcium ion by the sodium ion, and thus, the equilibrium between growth and detachment from the granules is shifted towards the decrease of the granules size. Furthermore, they remarked that at a high concentration of salt, no protozoa were found. As mentioned in Section 2.6, Weber, et al. (2007) state that ciliates might play a role in the formation of the EPS involved in the granulation. Therefore, the reduction of the protozoa population due to a high salt concentration, might have also affected the EPS strength and thus, lead to decrease of granules size.

Pronk, et al. (2014) state that contrary to suspended growth systems, in which nitrification is strongly affected by high salt concentration, in this study, ammonium removal was not affected in any of the concentrations of salt applied (0.2 to 20 g/L Cl^-). However, nitrite oxidation performance decreased when salinity was increased to 13 g/L Cl^- and further, registering a maximum nitrite concentration at the end of the cycle of 13 mg NO_2-N/L, whereas maximum nitrite concentration for 6.6 g/L Cl^- was 4.9 mg NO_2-N/L. Denitrification was lower at 0.2 and 20 g/L Cl^- than at 6.6 and 13 g/L Cl^-. Phosphate release was higher for 6.6 than for 0.2 g/L Cl^-, but it was completely inhibited at 13 g/L Cl^-, when phosphate uptake was also reduced.

CHAPTER 3

Materials and Methods

3.1. Laboratory activities

To accomplish the first two research objectives, two different reactors were studied during four months of operation: one AGS reactor and one AS reactor. The AGS reactor was installed at TU Delft Environmental Biotechnology laboratories, as part of the Ph.D. research of M.Sc. Danny de Graaff, who forms part of the research group of Mark van Loosdrecht. The AS reactor was installed at UNESCO-IHE laboratories.

These reactors were fed with synthetic wastewater containing *E. coli,* carbon source, and nutrients. Routine analyses consisted of COD, ammonia, phosphate, nitrate, nitrite, VSS/TSS, and *E. coli* to the influent, effluent, and sludge and supernatant in between the cycles. As a result, the performances of the reactors were followed, and removal efficiencies, removal mechanisms, and fate of *E. coli* were analysed. A scheme of the laboratory research methodology is depicted in Figure 3-1.

In the following paragraphs, a detailed description of the reactors, synthetic wastewater, sampling plan, analytical techniques, online measurements, and data analysis are given.

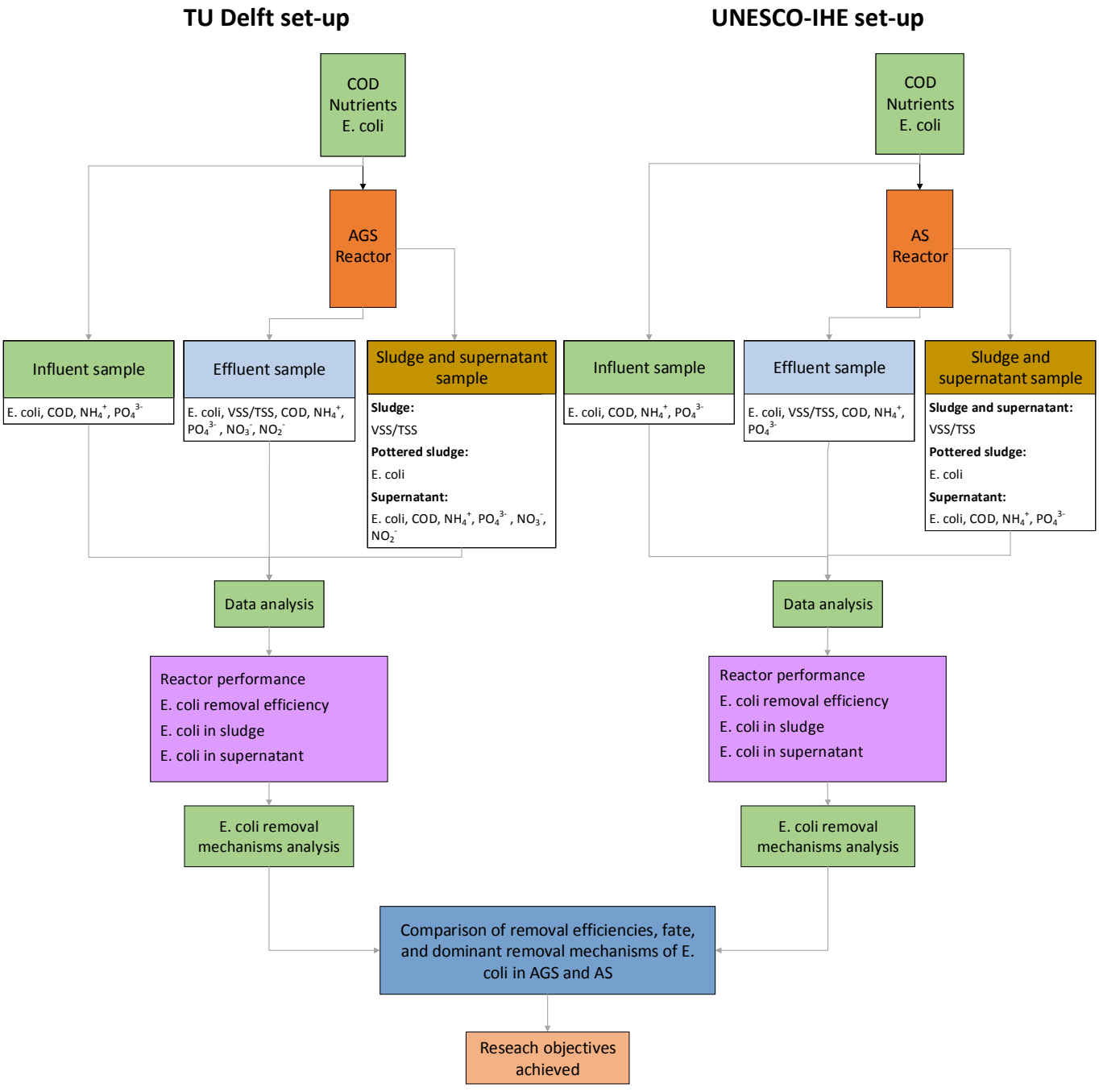


Figure 3-1: Scheme of laboratory research methodology

3.1.1. *Description of reactors*

AGS reactor

The AGS reactor used was a double wall glass column of 2.9 L of working volume, 60 mm in diameter, and 1,660 mm in height. This reactor replaced the one used during the first month of research which had a working volume of 2.7 L. The reactor operated as sequencing batch bubble column with four stages: 60 minutes of anaerobic plug flow feeding, 110 minutes of aeration, 5 minutes of settling, and 5 minutes of effluent discharge. Therefore, the length of the entire cycle was 180 minutes. The reactor was automatically controlled by a Bio-Controller Braun DCU4 coupled with mass flow control system and multi-fermentor control system (MFCS) 3 acquisition software (Sartorius Stedim Biotech S.A., Germany). The system had online measurements of temperature, DO, and pH.

The SRT was manually controlled at 20 days only after the first month of operation as it will be explained in Section 3.1.5. The Hydraulic Retention (HRT) was 5.8 hours. The seed sludge of the reactor was crushed granular sludge from the AGS line of Garmerwolde WWTP near Groningen, the Netherlands.

The influent synthetic wastewater consisted of three mediums and dilution water: COD medium, nutrients medium, and *E. coli* medium. The following concentrations were the theoretical results of diluting the mediums: 366 mg/L COD, 60 mg NH_4-N/L ammonium, 9 mg PO_4-P/L phosphate, 1 x 10^6 to 1 x 10^7 CFU/100 mL. The detailed composition of the mediums is presented in Annex 1. The *E. coli* was added to the reactor only after 1.5 months of operation when the granules were already formed. The influent mediums were diluted with tap water during the first three months of operation, and with demi-water during the last one due to a general measure taken for all reactors in the laboratory due to high copper concentration in the reactors. The mediums were placed in different bottles. The *E. coli* bottle was continuously stirred with a bottom stirrer to ensure homogenous distribution of the cells in the bottle, and covered with dark plastic to avoid growth of organisms.

The total influent volume was 1,500 mL. The mediums were supplied to the reactors with peristaltic pumps with a flow-rate of 150 mL/h. However, during the first 1.5 months of *E. coli* addition, the flow-rate of this medium was 600 mL/h; the bottle needed for this high flow-rate was extremely big, which made the bottom stirring unviable, therefore, during the last month of operation, this flow-rate was changed to 150 mL/h. The flow-rate for dilution water was 1,200 mL/h during the first 1.5 months, 600 mL/h during the second 1.5 month, and 1,050 mL/h during the last month. The tubes inside the pumps were Masterflex 14 tubes of 20 cm long. The connections between these tubes and the reactor were done with silicone tubes 3.0 x 6.0, 40 cm long (they had a small diameter to avoid algae growth). A one-way valve was located in the feeding tube which entered in the bottom of the reactor.

The aeration was provided through the bottom of the reactor by a diffuser with an air flow-rate of 6 L/min. Initially, the DO set point was planned to be in 20% of oxygen saturation (2 mg/L approximately), but due to decreased nitrification and denitrification performances during a period of operation, the DO set point was modified in order to favour these processes, as it will be explained further in this document. Nevertheless, the DO set point variations were

always between 20% and 50%. The set point was maintained by two mass flow controllers (one for compressed air and another for nitrogen gas); when DO was out of range, nitrogen gas was automatically injected. There was a gas recycle system to enhance the mixing.

The temperature in the bulk liquid of the reactor was 20°C, which was ensured by the double wall containing water at controlled temperature. The pH in the bulk liquid of the reactor was controlled between 6.6 and 7.1 by automatically injecting 1 M NaOH or 1 M HCl with peristaltic pumps whenever the pH was out of range. A volume of 1,500 mL of effluent was discharged from the middle of the reactor with a peristaltic pump. The volumetric exchange ratio of the reactor was 52%. The connection from the effluent pump to the reactor was materialized with a Masterflex 17 tube of 1 m long.

As mentioned before, some modifications in terms of DO, dilution water, and general operation of the reactor were performed in order to enhance its performance. In the following table, the actions carried out are summarized, which will be explained in Section 4.1.

Table 3-1: Actions taken to enhance AGS reactor performance

Actions	Date	Time of operation (days)
Set-up AGS reactor	01/11/2016	
Start-up AGS reactor	02/11/2016	0
DO from 20% to 50%	01/12/2016	29
DO from 50% to 20%	09/12/2016	37
New glass column installed	19/12/2016	47
E. coli feeding added	19/12/2016	47
DO from 20% to 50%	10/01/2017	69
DO from 50% to 20%	17/01/2017	76
DO from 20% to 50%	23/01/2017	82
DO from 50% to 20%	02/02/2017	92
Change from tap water to demi-water	02/02/2017	92
New *E. coli* pump of 150 mL/h	03/02/2017	93
Bottle of *E. coli* changed from 50 L to 10 L	03/02/2017	93
Change of DO probe	03/02/2017	93
DO from 20% to 50%	03/02/2017	93
DO from 50% to 20%	06/02/2017	96
DO from 20% to 35%	07/02/2017	97
DO from 35% to 45%	10/02/2017	100
DO from 45% to 40%	13/02/2017	103
DO from 40% to 35%	15/02/2017	105
DO from 35% to 45%	17/02/2017	107

Figure 3-2 shows a picture of the AGS reactor in the aeration stage at TU Delft laboratory and the detailed picture of the granules. In Figure 3-3 a scheme of the reactors' set-up is shown.

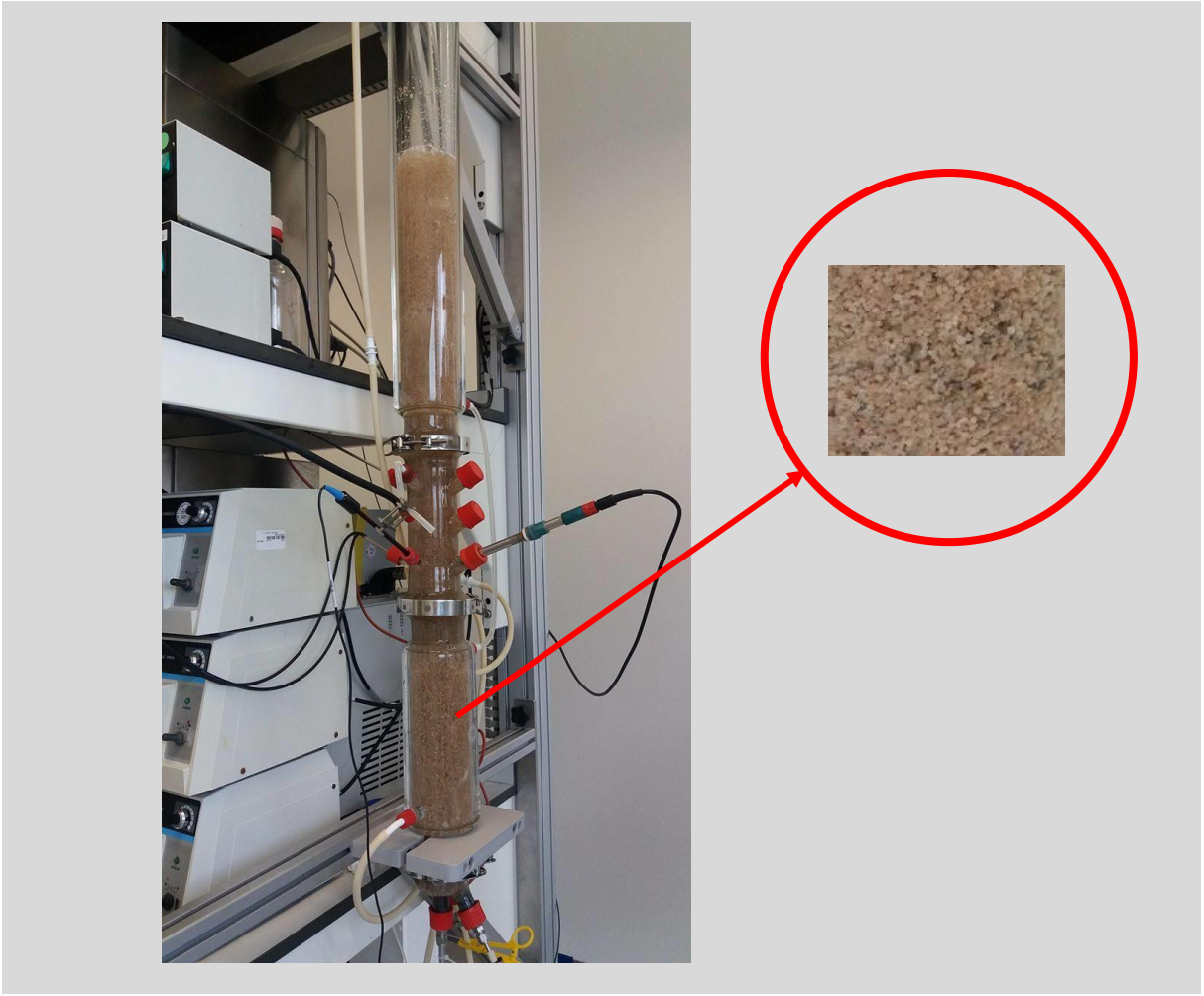


Figure 3-2: AGS reactor at TU Delft laboratory

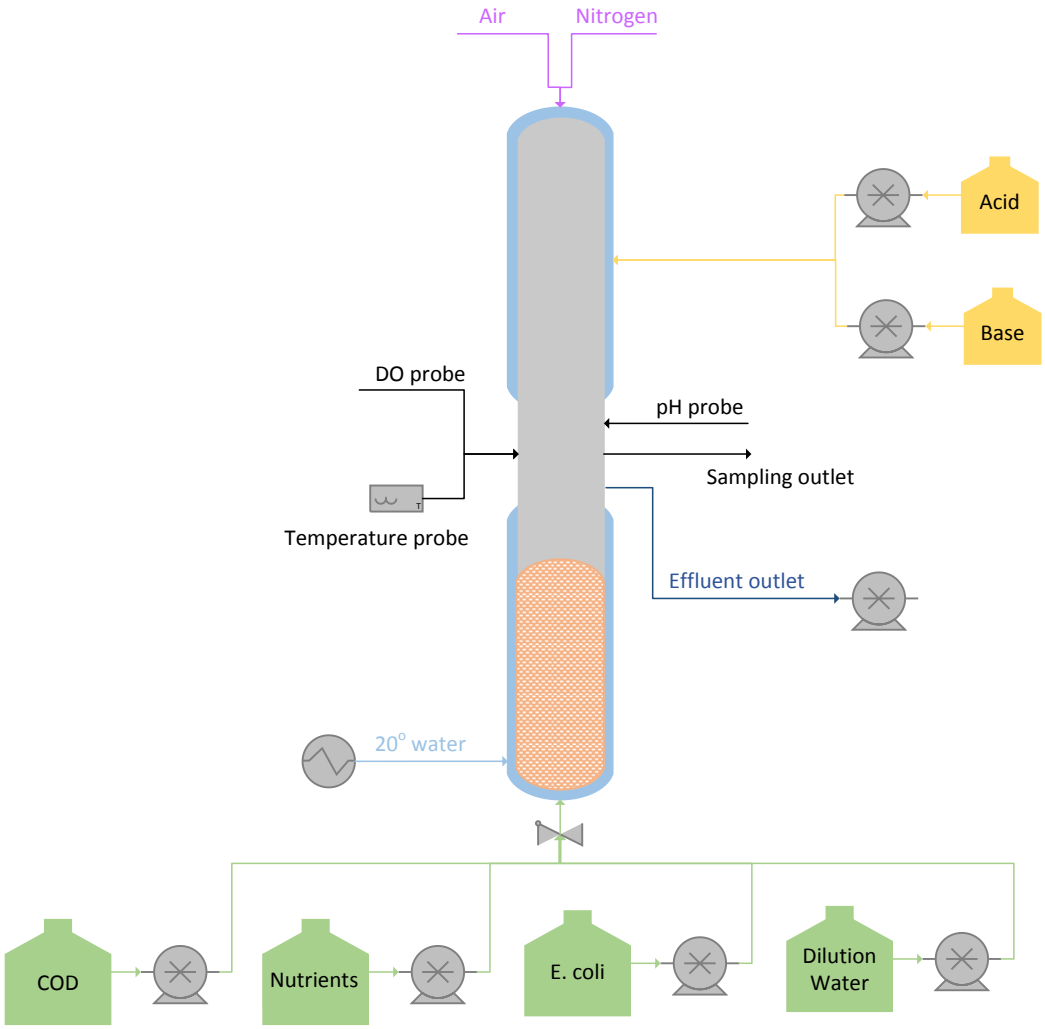


Figure 3-3: Scheme of AGS reactor

AS reactor

The AS reactor used was a double wall glass cylinder of 2.5 L of working volume. It was operated as SBR with a cycle designed for COD, nitrogen, and biological phosphorus removal. The configuration of the cycle varied during the research period in attempts to enhance the performance of the reactor, especially to favour nitrification, which was never achieved during the research period as it will be presented in Chapter 4. Nevertheless, the cycle consisted mainly of the following stages: anaerobic phase with feeding, aerobic phase, anoxic phase with COD feeding, refresh phase, WAS, settling, and effluent discharge.

The cycle was automatically controlled by a Bio-Controller ADI 1010 and a Bio Console ADI 1025 connected to a computer with the software BioXpert2. The system had online measurements of temperature, DO, pH, and acid and base injected volumes. The variations in the cycle were made adjusting the statements which controlled the SBR operation. The seed sludge of the reactor was sludge from the AS line from Garmerwolde WWTP, so it was originated from the same wastewater as the seed sludge for the AGS reactor.

The total influent volume was 1,250 mL. This reactor was fed with the same mediums as for the AGS reactor (Annex 1), but they were diluted with demi-water instead of tap water. As for the AGS reactor, the *E. coli* was added only after 1.5 months of operation, when the biomass was adapted to the synthetic wastewater and the flocs were already formed. The mediums were placed in different bottles. The *E. coli* bottle had the same characteristics as for the AGS reactor. The mediums were supplied to the reactors with peristaltic pumps.

The aeration was provided by compressed air. The temperature in the bulk liquid of the reactor was 20°C, which, as for the AGS reactor, was ensured by the double wall containing water at controlled temperature. The pH was automatically controlled, most of the time at pH 7, by injecting 0.4 M HCl or 0.4 M NaOH with peristaltic pumps whenever the pH was out of range. A volume of 1,250 mL of effluent was discharged from the middle of the reactor with a peristaltic pump. The volumetric exchange ratio was 50%.

In the following table, the characteristics of the initial cycle and of another cycle from an arbitrary day are presented. “sppH” means that there was pH control at the indicated set point; analogously for “spDO” and DO control.

Table 3-2: Initial cycle - AS

Phases	Anaerobic				Aerobic	Sampling	WAS	Settling	Effluent discharge	Stand by
Duration (min)	361									
	135				130	4	1	60	30	1
	5	5	25	100						
N_2 gas										
Stirring 500 rpm										
sppH 7										
COD 25 mL/min										
Nutrients 25 mL/min										
Demi-water 200 mL/min										
Compressed air										
WAS 33 mL/min										
Effluent discharge										
Desired SRT 20 days. HRT 12 hs. DO at 100% (9.1 mg/L). pH dead zone 0.05.										

Table 3-3: Cycle from day 84 to day 87 - AS

Phases	Anaerobic					Aerobic	Anoxic			Refresh	WAS	Settling	Effluent discharge	Stand by
Duration (min)	360													
	83					150	46			10	1	39	30	1
	5	3	2	20	53	150	2	22	22					
N_2 gas														
Stirring 500 rpm														
sppH 7														
COD 25 mL/min														
Nutrients 25 mL/min														
E. coli 40 mL/min														
Demi-water 160 mL/min														
Compressed air														
spDO 50%														
WAS 5 mL/min														
Effluent discharge														
Desired SRT 30 days. HRT 12 hs. pH dead zone 0.10.														

As an example, Annex 2 presents the statements correspondent to the cycle shown in Table 3-3. In the following table, the actions carried out to enhance the performance of the reactor are indicated, and they are explained in Annex 3.

Table 3-4: Actions taken to enhance AS reactor performance

Actions	Date	Time of operation (days)
Set-up AS reactor	01/11/2016	
Start-up AS reactor with Initial cycle	07/11/2016	0
Addition of anoxic phase	09/11/2016	2
Addition of anoxic feeding and refresh stage	10/11/2016	3
WAS corrected from 100 mL/cycle to 31 mL/cycle	25/11/2016	18
Chemical analyses at UNESCO-IHE instead of TU Delft	01/12/2016	24
Separation of COD pump	05/12/2016	28
DO control introduced at 22% with gas valve	05/12/2016	28
Decay experiments of *E. coli* feeding bottle started	05/12/2016	28

Actions	Date	Time of operation (days)
pH settings adjustment	07/12/2016	30
Gas valve removal	07/12/2016	30
Aeration duration increased from 100 to 150 min, and anaerobic duration decreased from 133 to 83 min	12/12/2016	35
Addition of fresh biomass	12/12/2016	35
SRT increased from 20 to 30 days by reducing WAS to 20 mL/cycle	13/12/2016	36
New start-up with fresh biomass	15/12/2016	38
E. coli feeding added	20/12/2016	43
WAS reduced to 5 mL/cycle, actual SRT 30 days	11/01/2017	65
DO increased from 22% to 50%	20/01/2017	74
Addition of alkalinity to the medium	01/02/2017	86
WAS pump disconnected	02/02/2017	87
Extension of cycle duration and DO increased from 50% to 70%	08/02/2017	93
pH set point decreased from 7 to 6.85, and dead zone increased from 0.10 to 0.30	14/02/2017	99
Mediums recipe corrected	17/02/2017	102
Anoxic phase shortened to 60 min	17/02/2017	102
Anoxic phase shortened to 46 min	23/02/2017	108
Dilution water changed to tap water	24/02/2017	109

Figure 3-4 shows a picture of the AS system at UNESCO-IHE lab.

Figure 3-4: AS system at UNESCO-IHE

3.1.2. *Maintenance of reactors*

Both reactors needed a preventive maintenance to minimize the probability of failures during the operation. This consisted of the complete clean-up of the reactors, replacements of the tubes when they seemed to have microbial growth or precipitations, and calibration of the pumps when replacing tubes. After a certain time of operation, it was also needed the refilling of the mediums, dilution water, acid and base bottles. The calibration of the pH and DO probes was done every two weeks.

Every week or every two weeks, the reactors were stopped and completely cleaned up. In the case of the AGS reactor, the clean-up consisted on placing the biomass temporarily in a beaker, disconnecting all the probes and tubes from the reactor, opening the top part of the reactor where a brush was introduced to remove all the possible material stuck to the walls, rinsing of the walls with demi or tap water (according to the dilution water used at that moment), and finally re-introduction of the biomass either from the top or with a funnel through a middle entrance. To clean-up the AS reactor, the head was removed and cleaned-up in the sink with tap and demi-water, the biomass stuck to the walls of the reactor was manually crushed and dispersed with gloves.

The calibration of the pumps was performed measuring the volume of liquid discharged by the pump in a beaker for different pump set points, until reaching the desired volume. Usually, it was done for 3 minutes or 5 minutes, the closer duration to the feeding duration, the more accurate calibration. The pH and DO probes calibrations were performed according to the standard procedures for each reactor.

3.1.3. *Sampling procedures and analytical methods*

The objective of the sampling and analyses was on one hand to follow the performance of the reactors, and on the other hand to study the removal and fate of *E. coli* in them. For this reason, weekly sampling and analyses of COD, ammonium, phosphate, VSS/TSS, and *E. coli* were performed for both reactors. Nitrates and nitrites were also measured for the AGS reactor. Furthermore, FISH analyses for *E. coli*, PAO's, and GAO's were also carried out. Also, samples were observed under the microscope for protozoa inspection.

Different samples were taken from the reactors during the entire period of operation. The sampling points were influent, effluent, and specific moments in between the cycle. For example at the end of the anaerobic phase (referred as end anaerobic henceforth) and at the end of the aerobic phase (referred as end aerobic henceforth). The sample representing the end anaerobic, was taken 2 minutes after aeration had started, for mixing purposes.

In order to sample the influent, the influent tubes were disconnected from the reactor, the pumps were turned on, and the influent was collected for 5 minutes in a beaker (except for *E. coli* samples as explained later in Section 4.1.3).

In order to collect the effluent samples, the effluent tube discharge was removed from its normal location (drainage) and placed in a bucket, where the effluent was collected. It is important to highlight that the effluent should be collected in one cycle, and the general sampling should be done in the following cycle, to avoid affecting the effluent sample in terms of TSS and amount of effluent discharged.

The samples in between the cycle were taken with a plastic syringe from the sampling point of the reactor. Sometimes these samples were homogenous, and sometimes they were manipulated to obtain more sludge and less supernatant (or vice versa) than a homogenous sample.

A detailed description of the sampling and analytical techniques used for both reactors is presented in the following paragraphs.

AGS reactor

Chemical analyses

Regarding the AGS reactor, the sampling points for the chemical parameters were the influent, effluent, and specific moments in between the cycle: minutes 60 (end anaerobic) and 90 on a weekly basis, and minutes 110 and 130 on a monthly basis. The sampling during aeration was done only in the first 70 minutes of this phase, because in previous experiences it had been noticed that the phosphate uptake mostly occurs during this period of time. All the chemical samples were filtered through Millex-HV 0.45 µm filters after sampling.

During the first month of operation only COD, ammonium, and phosphate were analysed; one month later, after the stabilization of the reactor, nitrate and nitrite were also included for the effluent and in between cycle samples. All the analyses, except for the influent, were performed at TU Delft with LCK cuvettes test (manufacturer: Hach). The influent analyses were performed at TU Delft during the first three months of operation and at UNESCO-IHE during the last month.

VSS/TSS analyses

The samples for VSS/TSS analyses were taken in duplicates from minute 60 and from the effluent. The analytical technique applied was a modification of the EPA Method 160.2. The approach of the TSS analyses regarding the samples in minute 60 was slightly different than usual. The sample had at least 10 mL of granules, and it did not need to be homogenous. Once the sample was taken, it was poured into a measuring cylinder, and the volume of the sludge bed was determined after 5 minutes of settling. After the TSS analysis, the results were extrapolated to the volume of sludge bed in the reactor. The effluent samples consisted on approximately 30 mL of homogenous sample; after collection they were weighted to determine the volume.

All the analyses were performed at UNESCO-IHE. All the samples were filtered through a Whatman glass micro fibre (GC/F) filter preheated in a muffle at 520°C for 2 hours. The filters were then dried at 105°C for 24 hours in aluminium cups. After drying, the weight of the filter and cup was determined (B); TSS of the sample was determined based on the difference between (B) and the empty weight of the cup and filter (A). After this, the filters and cup were heated at 520°C for 3 hours. The final weight was compared to (B) to determine VSS.

Plate counting

The samples for *E. coli* analyses were weekly taken after the addition of *E. coli* started, from the influent, effluent, minute 60, and minute 168 (end aerobic). Furthermore, before the addition of *E. coli* to the system, samples from influent, effluent, and end anaerobic were taken. During the first two sampling days, end aerobic was not sampled. In some specific weeks, samples in between the aeration were also taken to follow *E. coli* removal during this phase.

As the main objective of this sampling was to determine not only the removal of *E. coli* but also its fate, the granules and supernatant of the samples taken during aeration were analysed separately in order to determine the concentration of *E. coli* in each fraction. Once the sample was taken, it was poured into a slender plastic sampling cup, and the supernatant was removed after 5 minutes of settling (duration of settling phase in this reactor); the supernatant was poured into another sampling cup. The granules fraction was then pottered with a pottering tube to generate a homogenous sample.

In some specific weeks, a homogenous sample of pottered granules and supernatant was also analysed. This was done to compare the *E. coli* concentration of this sample with the respective fractions of pottered granules and supernatant analysed separately. Regarding the influent, the effect of the different durations of sample collection on the *E. coli* concentration was once studied.

Viable counts of *E. coli* of all the samples were performed in duplicates at UNESCO-IHE by the spread-plate technique using Chromocult coliform agar according to the procedure ISO 9308-1:2000 (ISO, 2000). The samples were diluted 10-fold, 100-fold, or 1000-fold with sterile peptone water (ISO, 2000) depending on the expected results (1 mL of sample in 9 mL of peptone water). After the dilution, 0.1 mL of the sample was spread in Chromocult coliform agar plates using sterile glass spreaders. After 24 hours of incubation at 37°C, the *E. coli*

colonies were identified as dark blue to violet dots and counted with a Colony counter. Other coliforms could be observed in pink colour and other Enterobacteriaceae in white to yellow colour.

Besides Chromocult coliform agar analyses, an aliquot of the samples was preserved to perform qPCR molecular analyses in the framework of the Ph.D. research of Mary Luz Barrios. In this way, the volume of each sample had to be enough for both analyses, in the case of influent and effluent, approximately 20 mL were taken; in the case of aeration samples, the sludge bed was preferably 15 mL and supernatant 20 mL or more.

While analysing some effluent samples, none of the dilutions showed growth of *E. coli* in the Chromocult coliform agar plates. This meant that *E. coli* concentration was below the detection limit of this technique, which is 10^3 CFU/100 mL in the case of plating 0.1 mL of undiluted sample. Thus, instead of assigning a value of zero in these cases, the detection limit was assigned.

In an attempt to find out which was the concentration of the samples in these cases, other methods with lower detection limit were experimented. For example, some trials were made spreading more than 0.1 mL of sample in the Chromocult coliform agar plates. It was realized that, in general, when spreading 1 mL, the amount of liquid was too much to produce clear results, sometimes the blue colour of *E. coli* was diffused, not forming a clear dot. When plating 0.5 mL or less, in general the results were clear. Also, some trials were made utilizing the medium Endo NPS. This technique consists first on filtering a certain amount of sample through a special filter which comes together with the Endo NPS pack, and later placing this filter in a dish with Endo NPS medium previously activated with 3 mL of sterile water. The dish is then incubated between 18 and 24 hours at 37°C. *E. coli* forms red colonies with a metallic sheen and red dots at the underside of the membrane. However, in general, this method did not show clear results, making the counting difficult.

The Chromocult coliform agar plates plated with undiluted pottered sludge samples, presented in general, small particles which sometimes interfered with the counting of *E. coli*. For samples with a low concentration of *E. coli*, the presence of particles was a problem, because the undiluted sample counting was not accurate because of the particles and the dilutions did not have enough colonies to consider the result reliable. Therefore, in order to get rid of these particles, the method “Microfiltration” was developed as presented in Annex 4. After finalising the development of this method, the amount of colonies obtained from the diluted samples with the spread-plate technique, was sufficient to consider the results as reliable, thus, the application of this method was not further needed.

During the process of determining which method was going to be used for separating sludge and supernatant, some trials of washing and centrifuging the sludge and supernatant were done with the first samples. Later on, it was decided that the method to be used was going to be just settling, because this would resemble the reality of a WWTP.

FISH analyses

As part of the Ph.D. research of Danny de Graaff, samples of the reactor were prepared and fixated for future DGGE and FISH analyses of the bacteriological community in the reactor.

The first month of operation, one sample per week was fixated with these purposes, later only one sample per month was fixated. The general steps for fixation are indicated in TU Delft protocol for FISH analysis, which is presented in Annex 5. Annex 6 presents a more detailed description of the preparation and fixation procedure for DGGE and FISH analysis elaborated by the author based on the training given by Danny de Graaff and Mary Luz Barrios.

During the last month of operation, samples of end anaerobic and end aerobic, of *E. coli* pure culture, and of a negative control of *E. coli* were fixated to perform FISH analysis targeting *E. coli*. The same month, *E. coli* FISH analysis was performed on these samples as part of a training given by Udo van Dongen at TU Delft. And in another opportunity, FISH analysis was performed on these samples at UNESCO-IHE targeting PAO's and GAO's as part of a training given by Francisco Rubio Rincón. The procedure followed for FISH analyses is presented in Annex 5.

The probes used for *E. coli* FISH were the following:

- EC 1531:
    - Target organism: *E. coli*
    - Formamide: 35%
    - Label colour: Cy3
- ECO1167 (ECO 45A):
    - Target organism: *E. coli*
    - Formamide: 40%
    - Label colour: Cy3
- Gamma 428:
    - Target organism: Gamma Proteobacteria
    - Label colour: Fluos
- EUB 338
    - Target organism: All bacteria
    - Label colour: Cy5

Two different mixtures of probes were analysed, first the mixture of EC 1531, Gamma 428, and EUB 338, and second, the mixture of ECO1167, Gamma 428, and EUB 338. The epifluorescence microscope utilized was Axioplan 2 (Zeiss), and the software to process the images was AxioVision version 4.8.

The mixture of probes used for staining PAO's and GAO's contained the probes PAO651 (Cy5) and GB (Fluos). Also, nucleic acid staining (DAPI) was added in this opportunity to stain all DNA present. The microscope utilized was Olympus BX51, and the software to process the images was cellSens Dimension.

Optical microscope observations

During the last 1.5 month of operation, samples of granules during aeration were observed to inspect protozoa presence under an optical microscope Olympus CH30 (4x, 10x, and 40 x) and a microscope Olympus BX51 (10x, 20x, and 40x) equipped with a camera. Pictures of the observations were taken.

In order to transform these observations into numbers, the variable “qualitative protozoa activity”, which indicates the protozoa activity based on qualitative observations under the optical microscope, was created. Based on the comparison of samples concerning the amount of protozoa present and the activity (mobility) shown by them, a value of 100% was assigned to the ones that showed more protozoa and more activity, and a value of zero was assigned if there were no protozoa present.

AS reactor

Chemical analyses

Regarding the AS reactor, the sampling points for the chemical parameters were the feeding bottles of COD and nutrients, influent, effluent, end aerobic, and in some opportunities also end anaerobic. All the chemical samples were filtered through Carl Roth GmbH + Co. KG 0.45 µm cellulose acetate syringe filters after sampling.

All these samples were analysed in terms of COD, ammonia, and phosphate. Nitrate and nitrite were not part of the routine analyses provided that ammonia results demonstrated that nitrification was not occurring. However, nitrate was sometimes analysed at end aerobic and in the effluent to confirm the absence of nitrification. During the first month of operation, the analyses were performed at TU Delft with LCK cuvettes test (manufacturer: Hach). After this, analyses were carried out at UNESCO-IHE. All the chemical parameters measured at UNESCO-IHE were measured spectrophotometrically. The COD analyses were performed according to Closed Reflux, Colorimetric Method; ammonia analyses were carried out in accordance with NEN 6472; phosphate analyses were done following the ascorbic acid spectrophotometer method; and nitrate was measured according to ISO 7890/1-1986 (Kruis, 2014).

The AS chemical analyses were performed in duplicates during the second and third month. However, the results of the duplicates were not always close enough to be considered a reliable result. This is due to the fact that in order to make the filtration of the sample easier, the sample was manipulated to obtain the less amount of solids possible, i.e. once the sample was taken, it was kept for approximately one minute in the syringe (which was upside-down still connected to the sampling point) to let the sludge settle, then the settled sludge was returned back to the reactor. Only after this, the duplicate could be taken. Therefore, there could be a time difference between duplicates, which might probably change the concentration of the parameters in both samples, since the bio-processes continued taking place in the reactor. Thus, on the last month of sampling, it was decided to take only one sample, and analyse it twice to quantify analytical errors.

VSS/TSS analyses

Samples for VSS/TSS analyses were taken in duplicates from the reactor at the middle of aeration and in the effluent. As explained before, the chemical samples were manipulated to obtain the less amount of solids possible, this means that these samples were not homogenous, and therefore, they slightly increased the solids concentration in the reactor. This was realized only on the last week of the sampling, therefore on the last sampling day, VSS/TSS reactor samples were taken before chemical samples, i.e. at the end of the anaerobic

phase. The analytical technique applied for the reactor and effluent samples was the same as for the effluent samples of the AGS reactor.

Plate counting

The samples for *E. coli* analyses were weekly taken after the addition of *E. coli* started, from the influent, effluent, end anaerobic and end aerobic. Furthermore, as for the AGS reactor, before the addition of *E. coli* to the system, samples from influent, effluent, and end anaerobic were taken. During the first two sampling days, end aerobic was not sampled.

As for the AGS reactor, the fraction sludge and the fraction supernatant were analysed separately. The method for the separation was the same as described before, but the settling time of the samples was 40 minutes, which coincided with the settling time of the cycle. Only one month after the sampling was started, it was decided to potter the sludge samples of this reactor, thus, the first samples of sludge were not pottered. A homogenous sample of pottered sludge and supernatant was also analysed for this reactor in some specific weeks.

As for the AGS reactor, all the samples were analysed in duplicates at UNESCO-IHE by the spread-plate technique as previously described. Also for this reactor, an aliquot of the samples was preserved to perform qPCR molecular analyses. The volumes of the samples were the same as for the AGS reactor. The particles observed in the Chromocult coliform agar plates of the AGS samples were also observed in the AS samples.

As for the AGS reactor, during the process of determining which method was going to be used for separating sludge and supernatant, some trials of washing and centrifuging the sludge and supernatant were done with the first samples. Later on, it was decided that the method to be used was going to be just settling, because this would resemble the reality of a WWTP.

During the first month of operation, a decay analysis of the feeding bottle of *E. coli* was performed in order to determine the maximum duration of the medium without decaying one order. Analyses were done using Chromocult coliform agar plates as described above.

FISH analyses

Samples of end anaerobic and end aerobic were fixated during the last month of operation. These were used in the same FISH analyses mentioned before for PAO's, and GAO's.

Optical microscope observations

The description presented for the AGS reactor is also valid for the AS reactor.

3.1.4. *Online measurements*

As mentioned before, pH, DO, and temperature were measured online in both reactors. Also, the addition of acid and base was recorded. Every week these profiles were observed to check if DO concentration was oscillating around the set points. The pH profiles were carefully checked, because they reflect the processes taking place inside the reactor. In the case of the AS system, at the beginning of the anaerobic phase a first rapid decrease of pH is expected due to phosphate release, then pH continues decreasing but slowly until phosphate release stops; at the beginning of the aerobic phase, there should be a rapid increase of pH followed

by a decrease due to nitrification, until reaching a plateau when all ammonia has been converted (Lee, et al., 2001).

3.1.5. *Data analysis*

Chemical and online measurements data analysis

The data resulting from the chemical analyses and online profiles of both reactors was analysed to evaluate their performances.

Regarding the AGS reactor, the resulting data from the chemical analysis and pH profiles was analysed to determine the most convenient value of the DO set-point in each case. When the ammonium in the effluent was too high or the pH profile was showing lack of nitrification, the DO set point was increased to favour nitrifiers. But at some point, the high DO was affecting denitrification, causing an increase of nitrate in the effluent. When nitrate was too high, DO was reduced. The analysis of the chemical data also included the calculation of the removal efficiencies for COD, ammonium, total nitrogen, and phosphate; and the removal rates for ammonium and phosphate.

In the case of the AS reactor, the changes in the cycle of the reactor, mentioned in Section 3.1.1, were introduced according to the results of these analyses and inspection of the online profiles. AS chemical analyses were performed in duplicates, thus, the results are averages of the duplicates; the standard deviation of them during the last month was an indication of the accuracy of the analyses. As part of the chemical data analysis, the removal efficiencies for COD, ammonium, and phosphate were calculated.

The equations used for the calculation of the removal efficiencies for both reactors, and removal rates for the AGS reactor, were adapted from Mosquera-Corral, et al. (2005). They are shown in the following paragraph, for a parameter A.

$$Removal\ efficiency\ (A)\ (\%) = \frac{CAi - CAe}{CAi} \qquad \textit{(Equation 1)}$$

$$Removal\ rate\ (A)\ \left(\frac{mg\ A/g\ VSS}{h}\right) = \frac{CA60' - CAe}{L \times VSS} \qquad \textit{(Equation 2)}$$

Where:

CAi: Influent concentration of A (mg A/L)

CAe: Effluent concentration of A (mg A/L)

CA60': Concentration of A in minute 60 of the AGS cycle (mg A/L)

L: Length of the aeration phase in the AGS reactor (h)

VSS: Volatile suspended solids (g VSS/L)

VSS/TSS data analysis

The VSS/TSS analyses results were used especially to control the SRT of the reactors. Taking into account the TSS in the reactor, TSS in the effluent, and the amount of sludge taken in each sampling, the solids exiting the reactor each week were calculated. Based on this, the extra sludge to take out of the reactor to achieve the desired total SRT was calculated. These calculations were based on the following formulas. Standard deviations of TSS measurements were also calculated.

$$Total\ SRT\ (d) = \frac{TSSr}{TSSos + TSSoe + TSSox} \times Sampling\ period \qquad \textit{(Equation 3)}$$

Where:

TSSr: TSS in reactor (g)

TSSos: TSS out with VSS/TSS samples (g/sampling period)

TSSoe: TSS out with effluent (g/sampling period)

TSSox: TSS out with extra samples and sludge removal (g/sampling period)

Sampling period: days between two samplings (d)

***E. coli* data analysis**

Plate counting

Regarding Chromocult coliform agar plates results, in general, three dilutions of the same sample in duplicates were spread-plated. In this way, there were six results per sample. According to Sutton (2011), the range of colonies commonly accepted in a plate as countable is between 25 and 250. Non countable plates were defined as those which colonies were not clearly countable because there was an excessive amount, or because of being too small in size, or because they were too close to other colonies. Plates showing no growth were assigned the detection limit, which as mentioned before was 10^3 CFU/100 mL in case of plating 0.1 mL of undiluted sample.

Once the plates were counted, a depuration of the results was done, choosing only the most representative results for each sample. The criteria to choose the most representative results was defined based on the fact that the fewer colonies in the plate, the more inaccurate is the result, because the same difference in number of colonies between duplicates would be more significant in percentage than in the case of a higher amount of colonies. Also, it was considered that if the duplicates were not similar in number, it meant that there was a mistake in the analysis of one of them, or in both. However, as they are duplicates and not triplicates, it cannot be determined which one is correct (unless one of them is comparable with another dilution). The defined criteria to choose the most representative results was the following.

1) If none of the dilutions had both plates with more than 20 CFU, the most representative was the less diluted.
2) If only one of the dilutions had both plates with more than 20 CFU, that was the most representative.
3) If more than one dilution had both plates with more than 20 CFU:
    a. If none of those had less than 20% relative error between duplicates, the most representative would be the less diluted.
    b. If only one of those had less than 20% relative error between duplicates, that was the most representative.
    c. If all of them had less than 20% relative error between duplicates, all of them were representative if they had 20% relative error between each other. If not, the most representative was the less diluted one.

After choosing the most representative results for each sample, the average of them and the standard deviation was calculated, assigning an *E. coli* concentration for each sample.

The formula for the relative error between a concentration C1 and a concentration C2 is the following.

$$Relative\ error\ (\%) = \frac{C1 - C2}{C1} \qquad \textit{(Equation 4)}$$

<u>*E. coli* log removal</u>

E. coli log removals were calculated using the following formula (Ríos, 2012) based on *E. coli* removal efficiencies, which were calculated with Equation 1.

$$E.coli\ log\ removal = Log\left(\frac{100}{100 - E.coli\ removal\ efficiency\ (\%)}\right) \qquad \textit{(Equation 5)}$$

<u>Logarithmic death of *E. coli* by ciliates predation</u>

As mentioned in Section 2.5, one individual of ciliated protozoa can filter certain volume of liquid per unit of time (F). Assuming that there is a fixed population density of ciliates in the reactor in one cycle (P), the total volume of liquid filtered by the ciliates in the reactor per unit of time is fixed in one cycle. Multiplying this volume by the concentration of *E. coli* in the reactor at that moment (C), the amount of *E. coli* cells removed per unit of time by ciliates can be estimated ((Co-C)/(t-to)). This can be expressed by the following formula.

$$\frac{(Co - C)}{(t - to)} = F\left(\frac{mL/h}{ind}\right) \times P\ \left(\frac{ind}{mL}\right) \times C\ \left(\frac{CFU}{mL}\right) \qquad \textit{(Equation 6)}$$

As stated before, F and P can be considered constant, therefore a constant k can be defined as follows:

$$k = F\ \times P \qquad \textit{(Equation 7)}$$

And therefore, Equation 7 can be re-written as follows.

$$-\frac{dC}{dt} = k\ \times C \qquad \textit{(Equation 8)}$$

Where C is the concentration of *E. coli* varying with the time t. This constant k is analogous to the Removal rate constant or Decay rate which is involved in the logarithmic death of microorganisms. Integrating the previous equation, the equation for the logarithmic death is obtained:

$$Ln(C) = Ln(Co) - kt \qquad \textit{(Equation 9)}$$

Taking into account the *E. coli* concentration at end anaerobic and at end aerobic, the *E. coli* logarithmic death curve during aeration can be determined. And therefore, k can be determined. Assuming that the only removal mechanism of *E. coli* is ciliates predation, knowing F, the population density of ciliates P in the reactor can be determined.

The Half-life of *E. coli* $t_{1/2}$ for the aeration period can be determined from k according to the following equation:

$$t_{1/2} = \frac{Ln(2)}{k} \qquad \textit{(Equation 10)}$$

Other experiments

The comparison between the concentration of *E. coli* in a homogenous sample (with a known volume Vs of sludge and a known volume Vsn of supernatant) and the concentration of *E. coli* in separate fractions of sludge and supernatant was done based on the following formula.

$$Cht = \frac{Cs \times Vs + Csn \times Vsn}{Vs + Vsn} \qquad \textit{(Equation 11)}$$

Where:

Cht: Theoretical concentration of *E. coli* in homogenous sample

Cs: Concentration of *E. coli* in sludge fraction

Csn: Concentration of *E. coli* in supernatant fraction

3.2. Methodology for the evaluation of AGS applicability in Uruguay

To accomplish the third and last research objective, which was the evaluation of the applicability of AGS treatment technology in Uruguay, two particular case studies were taken into consideration: Montevideo and Mercedes. In both cases, the characteristics of the wastewater and the Uruguayan standards will be compared to cases around the world in which AGS treatment technology is already successfully applied.

CHAPTER 4

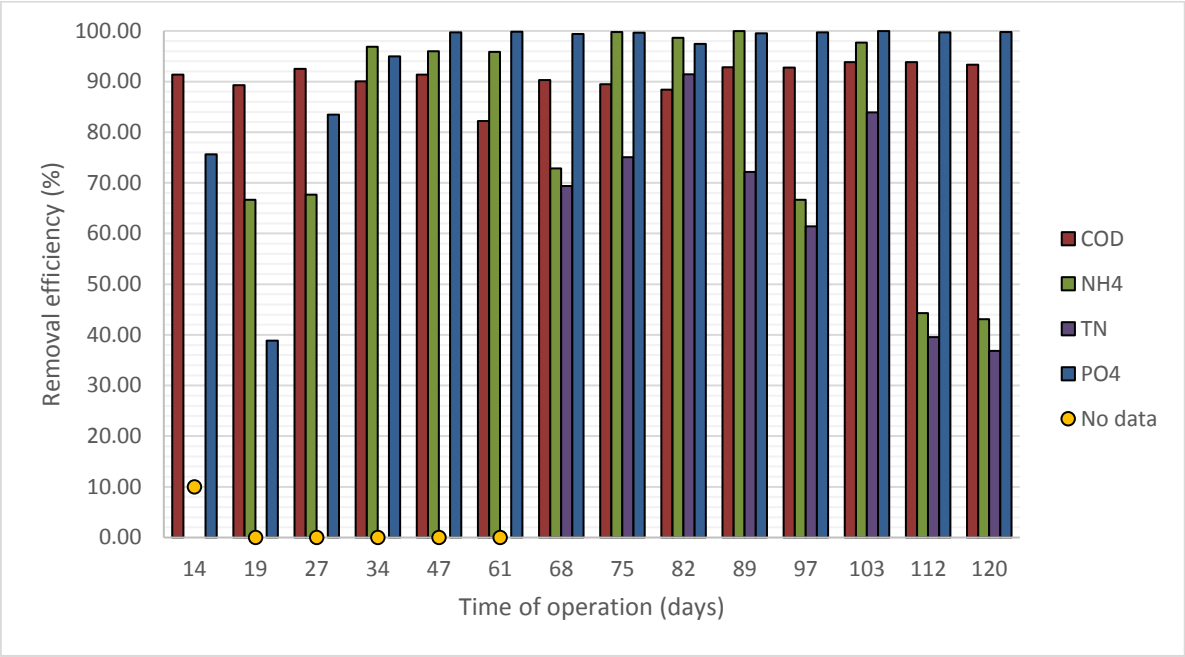
Results and Discussion

The results of the analyses and observations for the AGS and the AS reactors and for the evaluation of AGS applicability in Uruguay, are presented in this chapter.

4.1. AGS Reactor

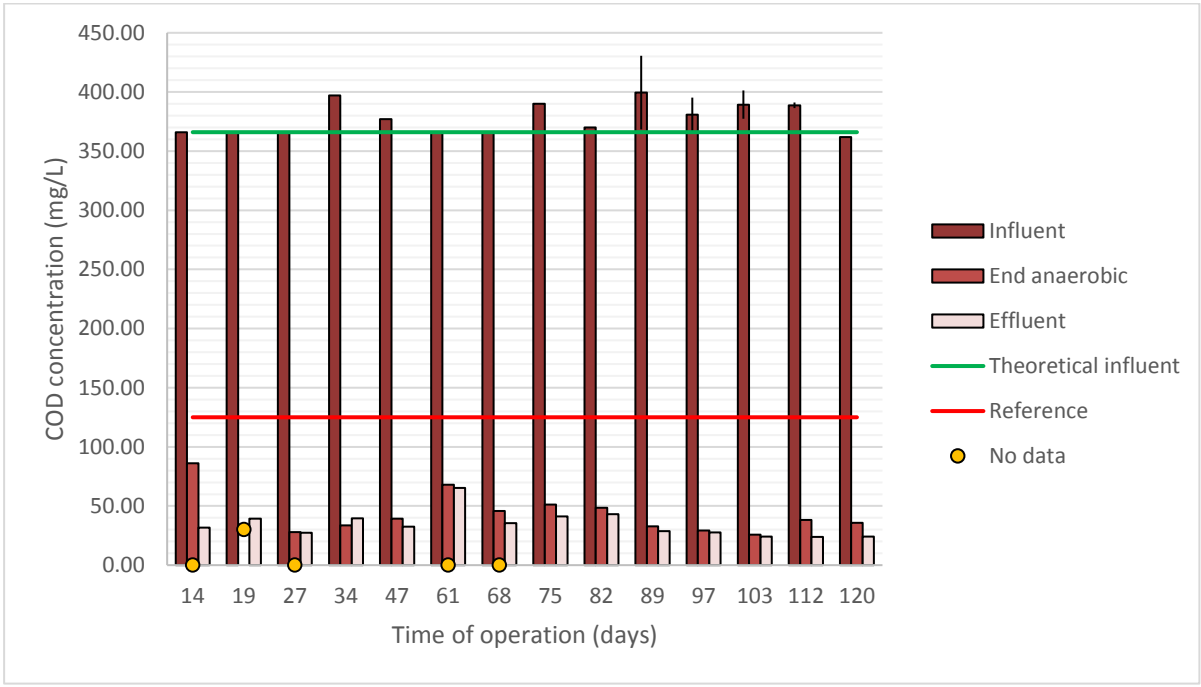
4.1.1. *Organic matter and nutrients removal*

The removal efficiencies for COD, ammonium, phosphate, and total nitrogen; the results for the analyses of COD, ammonium, and phosphate for influent, end anaerobic, and effluent; and the results for nitrate, nitrite, and total nitrogen for effluent; all of them for the different days of operation of the reactor, are presented in the following graphs and in Annex 7. The theoretical concentrations of COD, ammonium, and phosphate in the influent is indicated in the graphs. Also, reference values based on the EU standards for the discharges of urban wastewater treatment plants are indicated (Directive, 1991). The missing data is indicated as "No data"; in the case of influent concentrations missing, the theoretical value is indicated instead. For a particular sampling day, if the data of only one sampling point is missing, a value of zero was assigned to the variable "No data", but when data for more than one sampling point is missing (e.g. influent and end anaerobic), a value different from zero was assigned to the variable. The standard deviations of the influent are also shown in the graphs.



Graph 4-1: Removal efficiencies of organic matter and nutrients – AGS

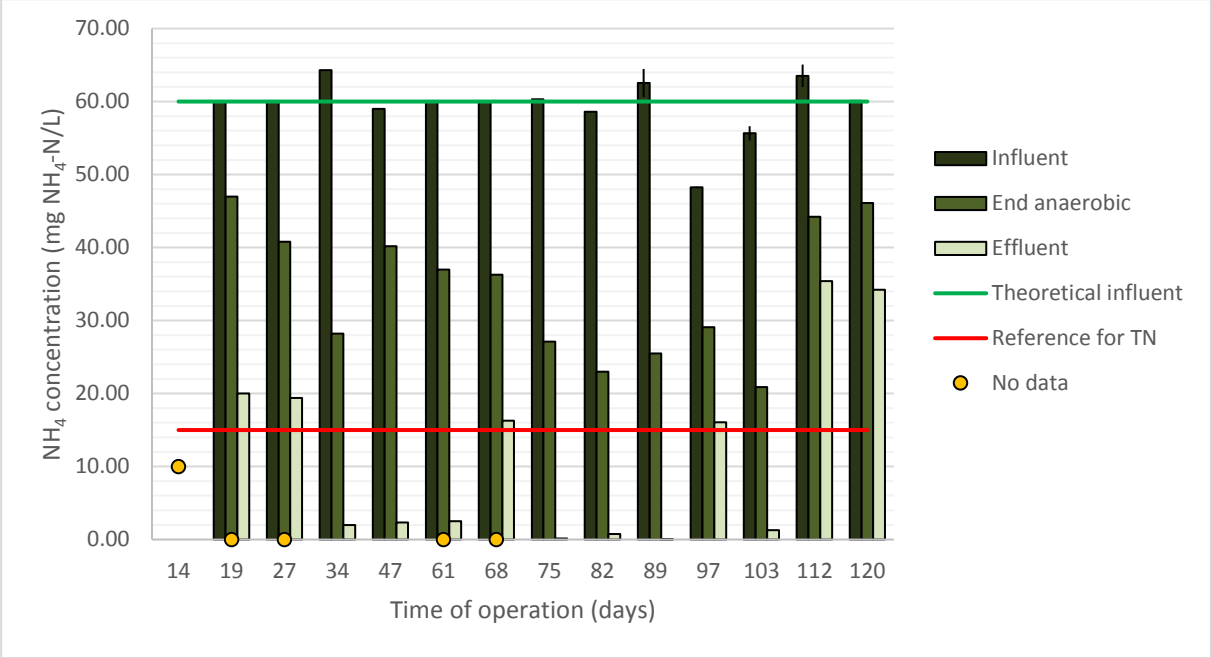
In the previous graph, it can be observed that the removal of COD was around 90% during the entire sampling period. Regarding the removal of ammonium, it is above 90% in more than 50% of the sampling days. The removal of total nitrogen oscillates between 40% and 90%. The removal of phosphate is excellent, reaching 100% since day 47 onwards.



Graph 4-2: Removal of COD - AGS

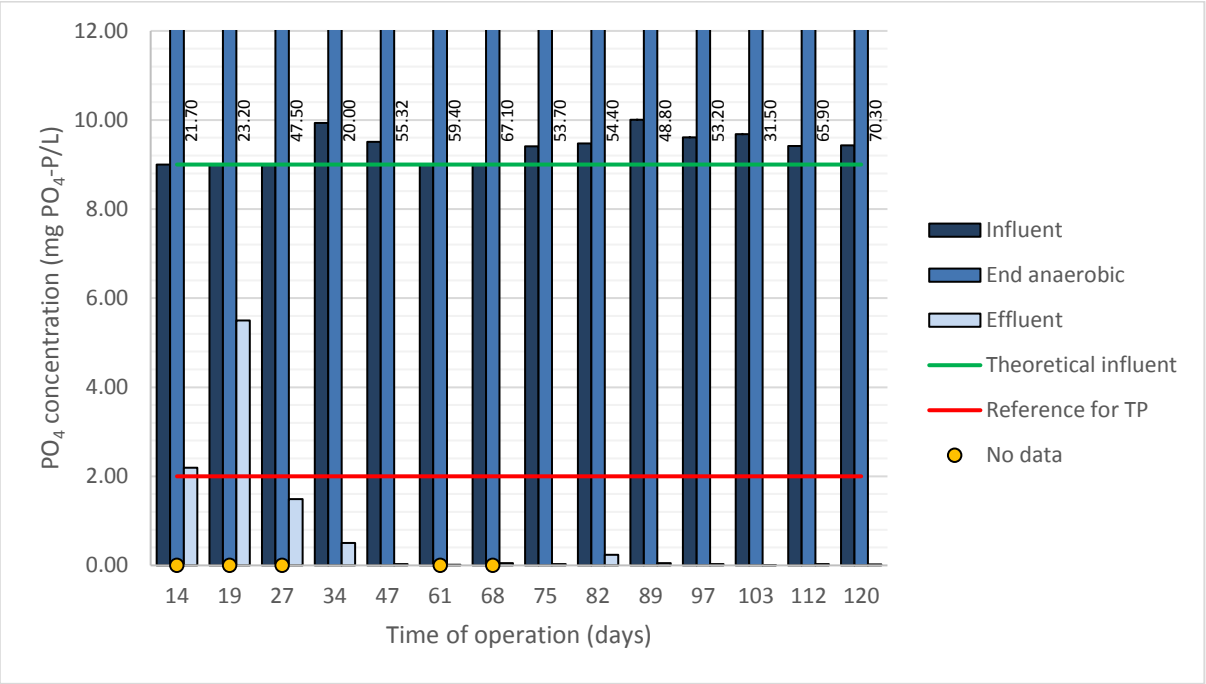
As it can be observed in the previous graph, from day 27 onwards, COD removed is almost entirely consumed in the anaerobic phase. Furthermore, the effluent is always below the

reference value. The minimum concentration achieved for COD in the effluent of 23.9 mg/L can be attributed to the fact that, according to De Kreuk, et al. (2010), the EDTA contained in the nutrients medium is not biodegradable. De Kreuk, et al. (2010) used the same trace metal solution at the same proportion as in this research, and they concluded that the concentration of COD in the effluent of 28 mg/L corresponded to the EDTA. According to an expert source (Danny de Graaff, personal communication, 13th December 2016), this is usual in AGS reactors, and in these cases, no acetate is found in the effluent when measured. Therefore, the real average removal efficiency of COD would be around 100%.



Graph 4-3: Removal of ammonium - AGS

The removal of ammonium during the aeration phase corresponds mainly to the nitrification process occurring in the granule. It can be observed that there was a fluctuation of nitrification performance; e.g. on days 34 to 61 a good nitrification is shown, whereas for the last two samplings, nitrification was not occurring. This can also be explained via removal rates; for days 34 to 61 the average removal rate of ammonium during aeration was 3.07 mg N/g VSS/h, while the average for the last two samplings was 1.28 mg N/g VSS/h. The maximum removal rate of the entire period corresponds to day 47 with 3.16 mg N/g VSS/h, and the minimum one corresponds to day 112 with 0.97 mg N/g VSS/h. The effluent is above the reference value when nitrification was not being achieved. More details about nitrogen removal are shown in Graph 4-5.

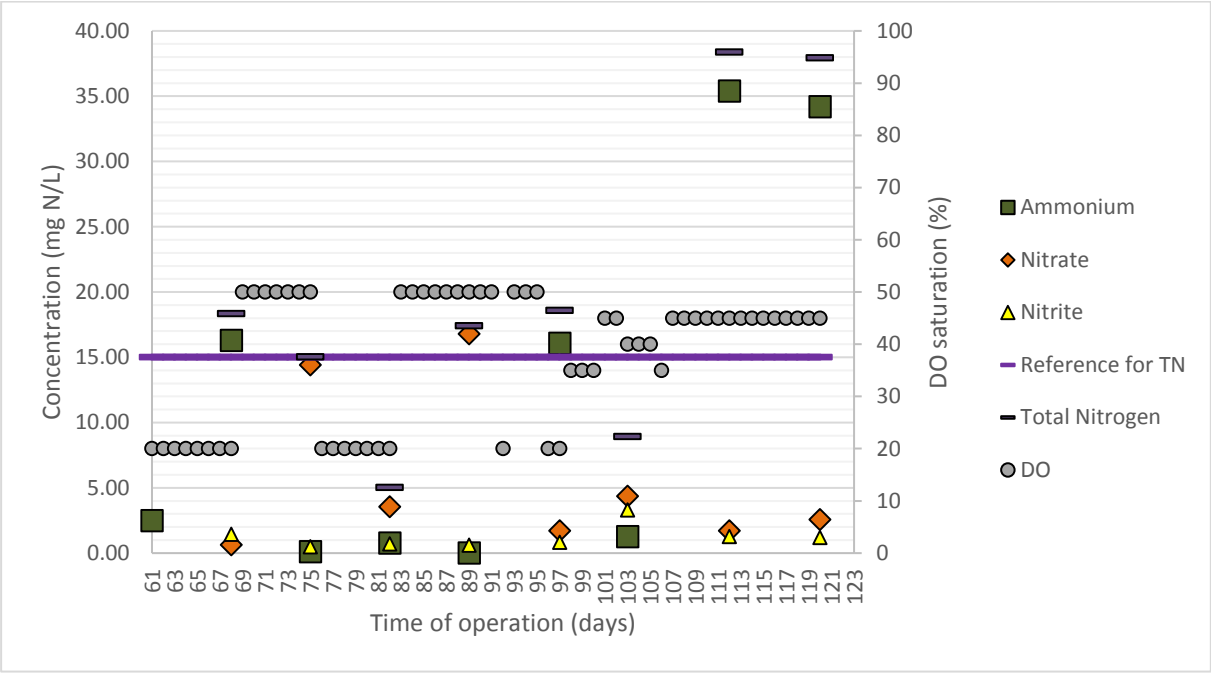


Graph 4-4: Removal of phosphate - AGS

From day 27 onwards, phosphate removal during the aeration period was excellent; from day 47 onwards, the effluent is below one quarter of the reference value, showing a good performance of the PAO's. The high concentration of phosphate at the end anaerobic indicates a great amount of phosphate released during the anaerobic period, which also demonstrates the good performance of the PAO's. The average removal rate during the aerobic period is 4.83 mg P/g VSS/h, being 2.24 mg P/g VSS/h the minimum (day 34) and 8.18 mg P/g VSS/h the maximum (day 120).

Based on the four graphs presented above, it can be observed that from day 14 to day 27 approximately, the performance of the reactor was not yet stable, this corresponds to the first month of operation, when the granules were being formed and the biomass was getting adapted to the synthetic wastewater.

As mentioned before, the DO concentration was modified to enhance simultaneous nitrification and denitrification based on the results of the chemical analyses of ammonium and nitrate in the effluent, on the observations of the pH profiles, and sometimes also based on the macroscopic observation of the granules. The range of DO variation was always between 20% and 50%. As the routine measurement of nitrate and nitrite only started at day 68, in the following graph the concentrations of ammonium, nitrate, nitrite, and total nitrogen in the effluent from day 68 onwards are presented. Also, the DO concentration and a reference value for total nitrogen for each day are presented. In Figure 4-1 the pH profile for days 92 and 93 is shown.



Graph 4-5: Nitrogen – Effluent AGS

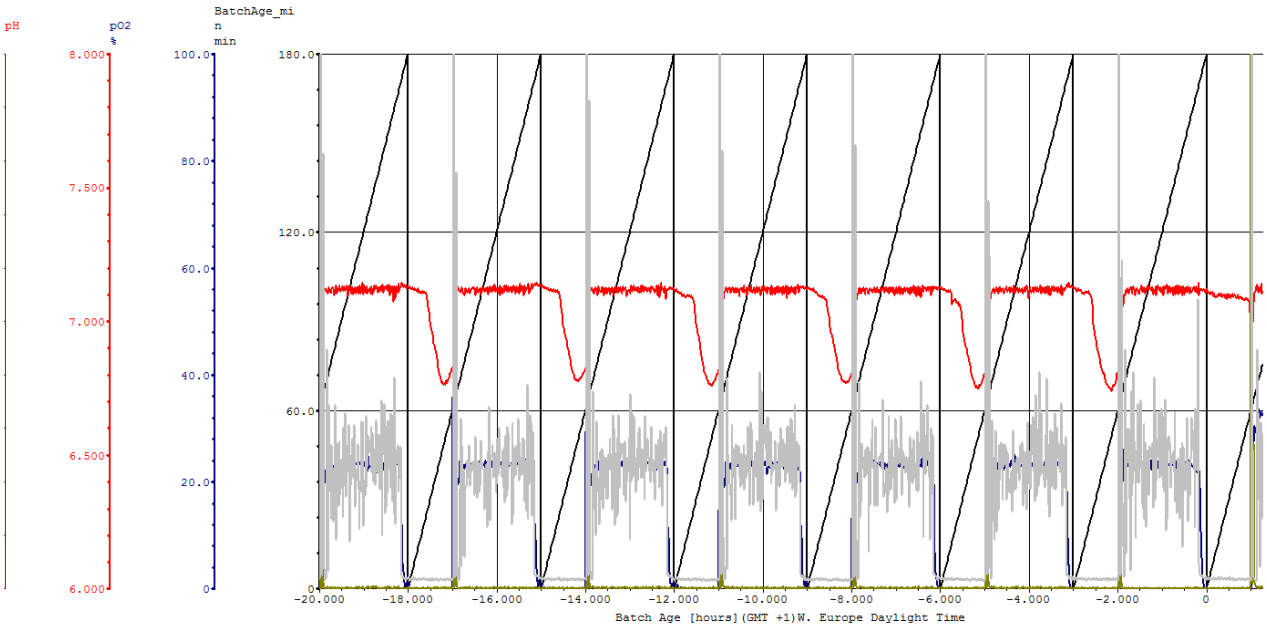


Figure 4-1: pH profile (red) days 92 and 93 (20% DO)

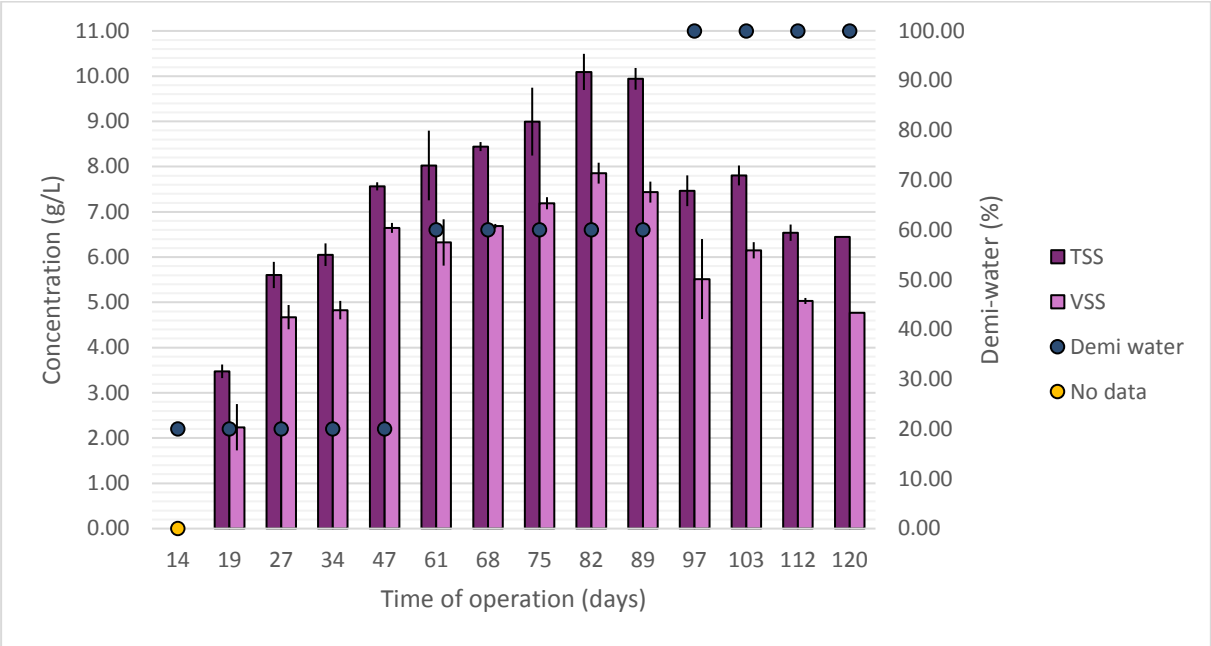
In Graph 4-5 it can be observed that on day 68, DO was increased from 20% to 50% after the increase of ammonium in the effluent, which indicated a decrease in the nitrification. The next sampling day, ammonium was low, but nitrate was high, which indicates a deterioration in the denitrification since the anoxic zone might have been reduced due to excess of DO, therefore, DO was reduced again. Concentrations of ammonium and nitrate on day 82 were satisfactory, but the presence of black granules which indicated lack of oxygen was macroscopically noticed, therefore, on the basis of an expert suggestion (Mario Pronk, personal

communication with Mary Luz Barrios, 23rd January 2017), the DO was increased again to 50%. After this, on day 89 nitrate was high again, so DO was reduced on day 92. But only for one day, because on day 93, the pH profile presented in Figure 4-1 was showing no pH variation during aeration, i.e. no nitrification was taking place, thus, oxygen was increased again. The same criteria was applied for the following DO variations. The cause of lack of nitrification during the last two weeks despite the high concentration of DO of 45% could not be found. Calibration of DO probe and the actual concentration of DO entering the system were checked: it was confirmed that the measured value by the DO probe in the reactor while aerating (100%) was the same as the measured value by the probe in an aerated bucket with water.

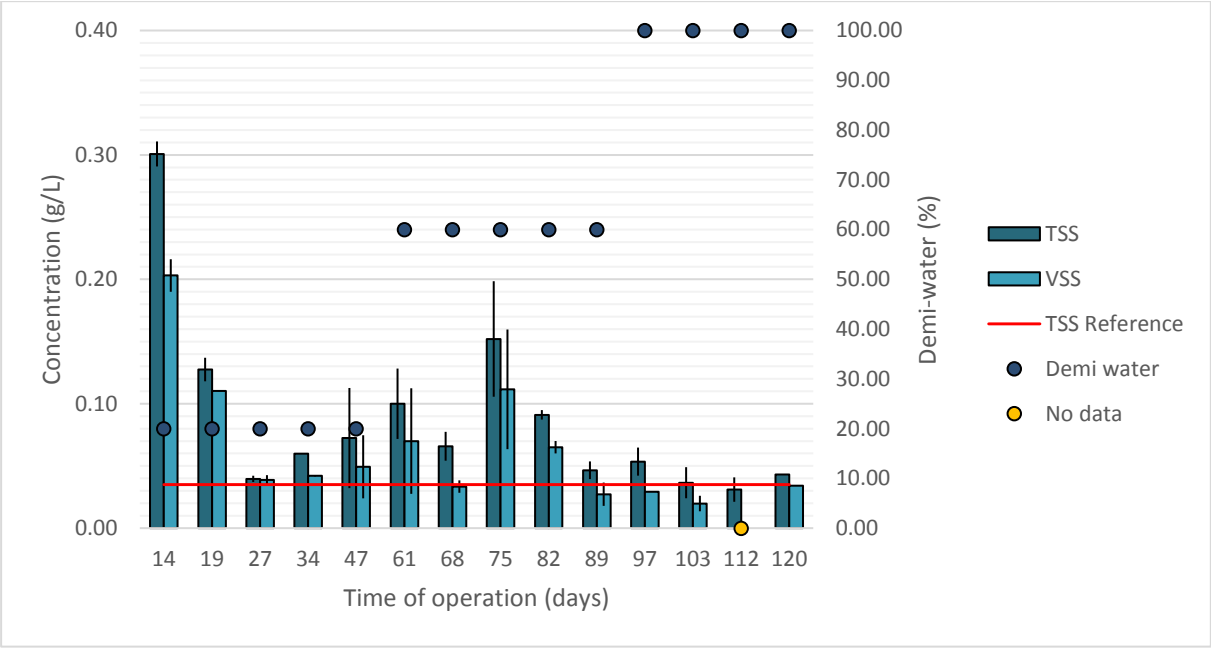
FISH analysis was performed to check the presence of PAO's and GAO's in the AGS reactor with probes which are proved to work with these organisms. The analysis was done on samples of pottered sludge taken on the last month of operation of the AGS reactor during end aerobic. The used probes were PAO651 and GB as presented in Section 3.1.3. Also, DAPI was added. The resulting pictures are presented in Annex 8. The quality of the pictures is not very good due to the fact that this analysis was part of a training, and therefore the analysts did not have expertise neither in hybridization nor in acquisition and processing of the images. However, it is possible to identify GAO's and PAO's, and therefore, it can be concluded that both organisms were present in the AGS reactor.

4.1.2. *VSS/TSS*

The results for the VSS/TSS analyses of the reactor and effluent, and the resulting SRT from performing SRT control at 20 days, are presented in the following graphs. The VSS/TSS graphs also present the standard deviation of the measurements, reference values based on the EU standards for the discharges of urban wastewater treatment plants (Directive, 1991), the missing data as "No data", and the demi-water proportion with respect to the total dilution water in each sampling day. Results for VSS/TSS are also presented in Annex 7. Besides the resulting SRT for each week, the SRT graph also shows the desired SRT for each week (20 days), the volume of the sludge bed of the reactor, and the volume of sludge taken from the reactor each week due to sampling, SRT control, or sludge bed control.



Graph 4-6: Reactor VSS/TSS – AGS



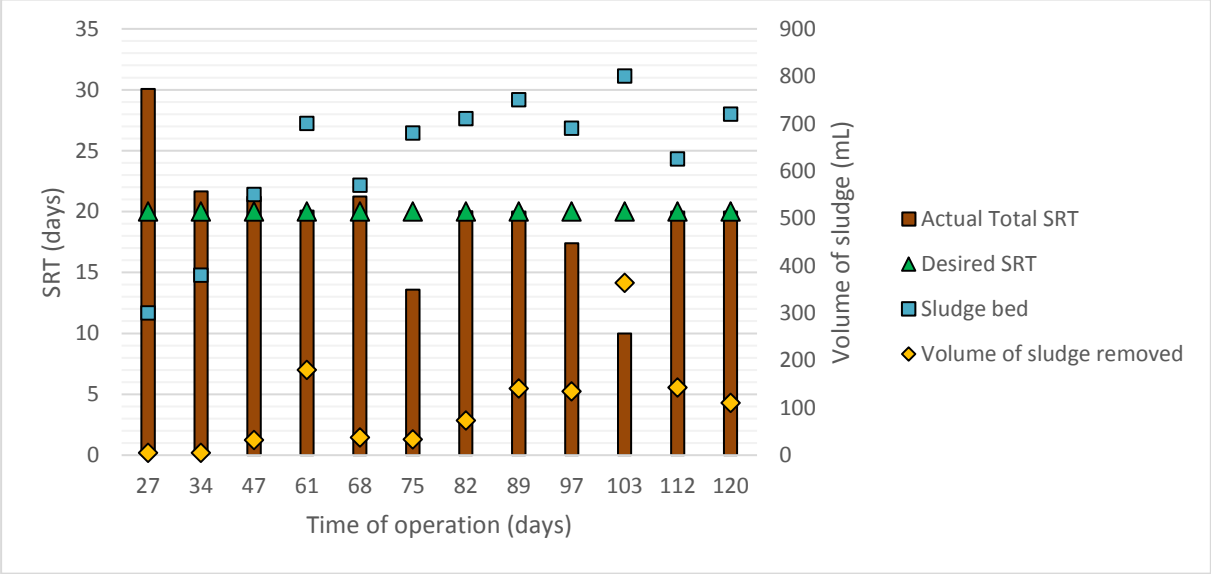
Graph 4-7: Effluent VSS/TSS – AGS

As it can be observed in Graph 4-6, TSS in the reactor was steadily increasing until day 82, reaching 10 g/L, when it started decreasing. From day 89 to 97 there is an abrupt decrease. Between these two sampling days, two relevant changes were performed: the dilution water was changed to 100% demi-water as it can be observed in the graph, and DO started to be modified because of decrease of nitrification and denitrification performances. As mentioned before, dilution water was changed to 100% demi-water because of high copper concentrations in the reactors found during the last weeks of the third month of operation.

Therefore, this abrupt decrease might be related with any of these events, or even with other factors, like the fact that the granules were getting too big, and due to this, they suddenly broke. This last hypothesis cannot be confirmed because the size of granules was not measured during the entire period of research.

Regarding TSS in the effluent, a trend was not found, it fluctuates within the entire research period. Furthermore, values before day 61 are not as accurate as after it. This is because before this day, the effluent sampling was done after the rest of the samplings, and as mentioned before, this might affect the amount of solids in the effluent if the samples were not homogenous. Taking into account the measurements between day 61 and 120, the average effluent TSS was 0.069 g/L. Day 112 is the only one complying with the reference value.

The average VSS/TSS ratio in the reactor is 78%, and 69% is for the effluent.



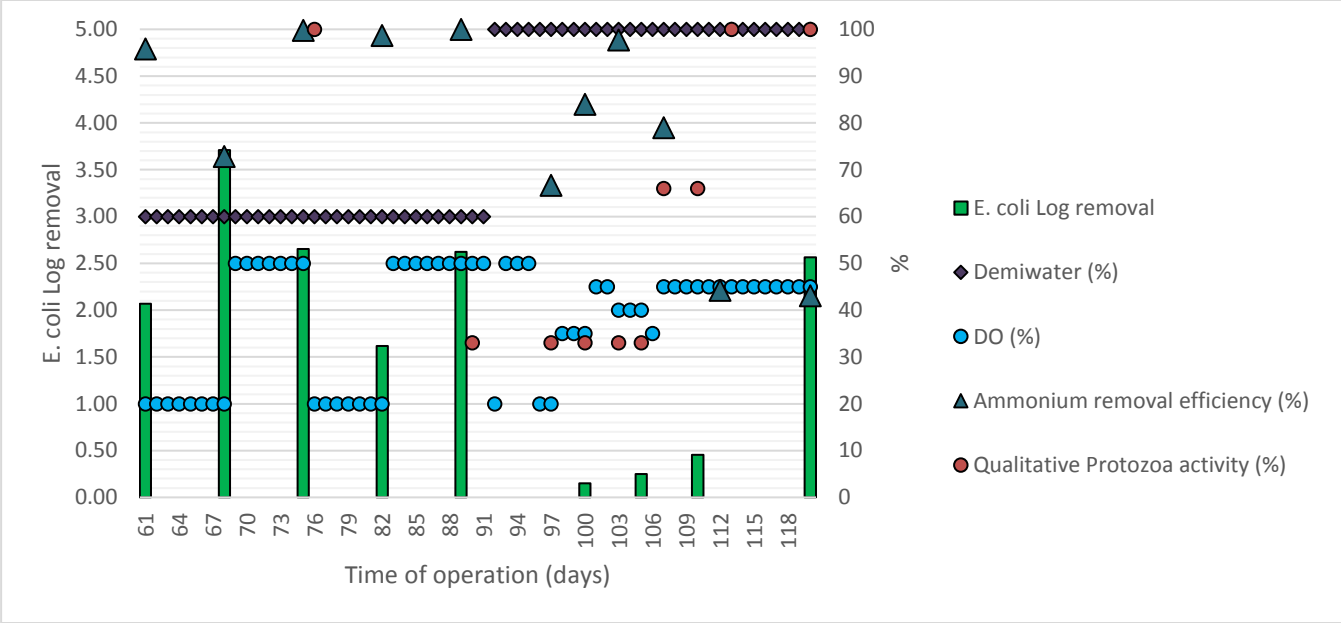
Graph 4-8: SRT - AGS

The previous graph shows that there was a good control of the SRT, being almost always in the desirable value, except for days 75, 97, and 103. The SRT of day 75 is low because despite the volume of samples taken was not significant (around 30 mL), TSS in the reactor and in the effluent were high, so less amount of sludge than usual needed to be taken that week to achieve 20 days of SRT. The SRT of day 97 is slightly lower than 20 days because of the large amount of samples taken and of the effluent TSS being higher than the previous week. In the case of day 103, the calculated SRT is low (10 days) because, apart from the sampling, 240 mL of sludge were removed due to the fact that the sludge bed in the reactor was too high, there were 800 mL sludge bed, and it is recommended to have between 400 and 500 mL (sludge bed control). As it can be observed in the graph, for unknown reasons, the sludge bed increased 100 mL in only one week, when it was usually increasing approximately 40 mL or even decreasing depending on the volume of samples taken the previous week.

4.1.3. *E. coli removal*

E. coli log removal

The log removal of *E. coli* taking into account influent and effluent *E. coli* concentrations is presented in the next graph and in Annex 7. The demi-water proportion, DO saturation, ammonium removal, and qualitative protozoa activity are also indicated.



Graph 4-9: Log removal of E. coli - AGS

In the previous graph, it can be observed that the log removal of *E. coli* in the AGS reactor is not stable. On day 61, after two weeks of starting *E. coli* addition in the reactor, 2 log removal of *E. coli* is shown. After this, the maximum removal of the entire research period which was 3.5 log removal, is achieved. Log removal starts decreasing and increases again on day 89. The log removal was above 1.5 for the first five weeks, presenting a maximum of 3.5 log removal and an average of 2.5 log removal. Day 97 shows no removal, so the sampling was repeated on day 100, resulting also in an insignificant removal. After this, the removal starts slowly increasing again, until achieving on day 120, the same removal as on days 75 and 89.

Protozoa presence

As shown in the previous graph, protozoa were found in the samples of granules during aeration which were observed under the optical microscope. The genus *Vorticella* spp., which is a sessile (stalked) ciliate was mainly identified. On days 76, 113, and 120 there were plenty of *Vorticella spp.*, very active, moving and eating. In the case of days 107 and 110, protozoa were not so active, and there were fewer individuals. Finally, between days 90 and 105, protozoa were hardly found in the samples. The following pictures show the *Vorticella spp.* observed in different samples.

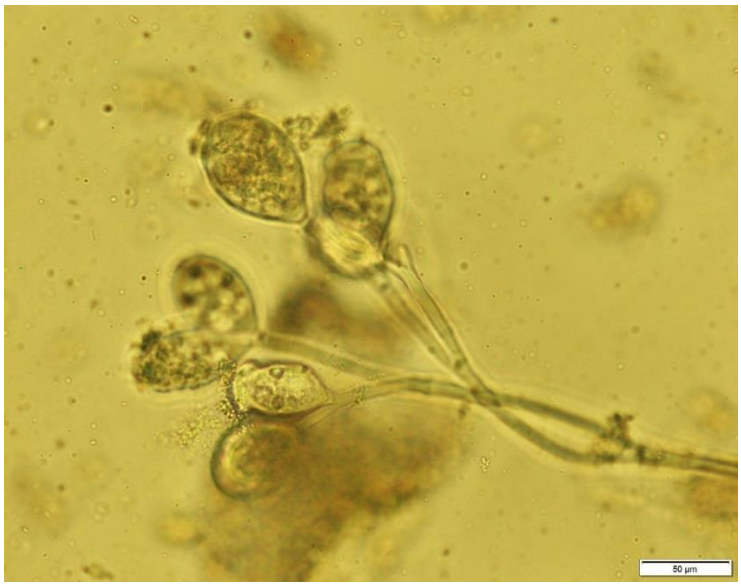


Figure 4-2: Optical microscope snapshot (20x) showing Vorticella spp. in granules of AGS reactor – Day 76

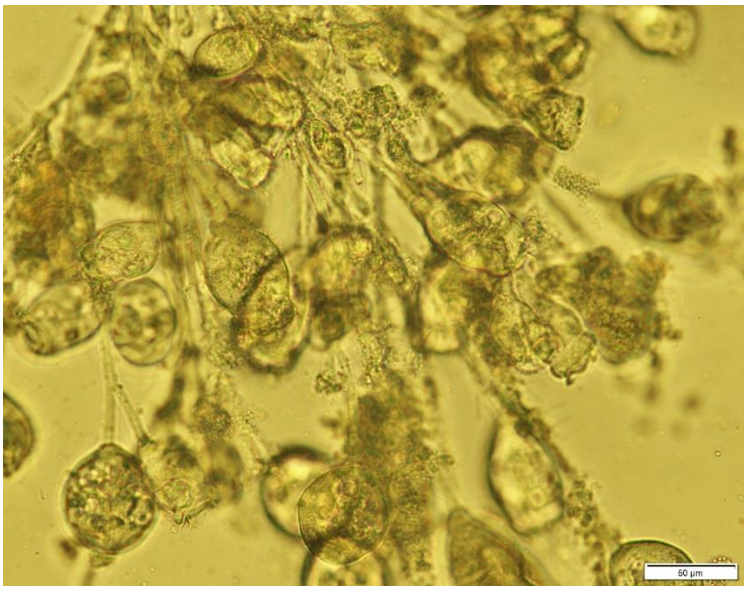


Figure 4-3: Optical microscope snapshot (20x) showing Vorticella spp. in granules of AGS reactor – Day 76

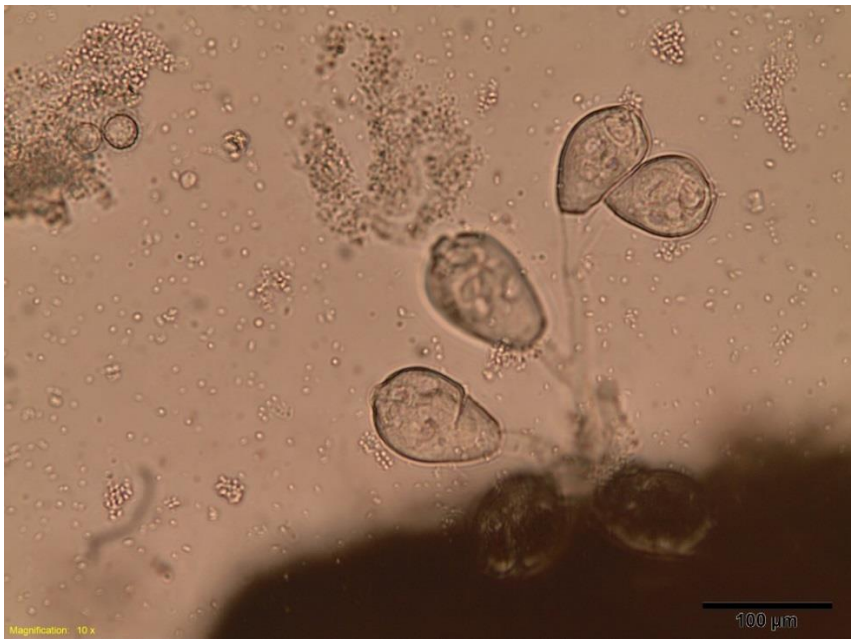


Figure 4-4: Optical microscope snapshot (10x) showing Vorticella spp. in granules of AGS reactor – Day 89

Other protozoa, such as free swimming ciliates were also found, but the predominant genus was *Vorticella*. As mentioned in Section 2.5, ciliates predate on bacteria. Stalked ciliates have less energy requirements than other classes of ciliates, and they dominate when the number of bacteria available for predation is less than the required for free-swimming ciliates. Therefore, if the number of bacteria available for predation was higher, more free-swimming ciliates might have been found. As stated in Section 2.5, when bacteria grow collectively forming flocs or biofilms, they protect themselves from being predated due to the fact that they form aggregates of bigger size than the edible particles by protozoa. Thus, in this case, the available bacteria for ciliate predation were the free-suspended bacteria, excluding bacteria forming granules.

As mentioned in Section 2.4, ciliates predation is the main removal mechanism for *E. coli* in AS, therefore, a correlation between protozoa presence and removal of *E. coli* was expected to be found. One of the causes of the decrease in *E. coli* removal from day 89 to 97 might have been the reduction of protozoa activity, i.e. the inhibition of protozoa. Indeed, qualitative protozoa activity on day 90 was less than on day 74, and it stayed low until *E. coli* removal started increasing. However, this correlation cannot be confirmed with this research because the population density of ciliates was not measured.

The stated correlation between the number of ciliates and the *E. coli* removal could be further studied adding as a routine measurement the quantification of ciliates, for example with light microscopy as described in Li, et al. (2013), or with qPCR. Furthermore, experiments aimed at artificially increasing or reducing the number of protozoa, to observe the effect on *E. coli* removal can be done. Different techniques have been proven to affect the population density of ciliates. According to Pauli, et al. (2001), the addition of sodium azide or sodium fluoride to a typical AS microbiological community reduce the protozoa population density; they also mention that ciliates population was declined when bench-scale AS plants were exposed to

temperatures higher than 36°C. On the other hand, they state that the addition of emulsified lipids to bench-scale AS plants leads to a fast increase of stalked ciliates population. De Kreuk, et al. (2010) proved that the addition of particulate starch to an AGS reactor provoked an excessive growth of stalked ciliates (mainly *Vorticella* spp and others) and rotifers. However, this also led to the proliferation of filamentous organisms attached to the granules, resulting in granules with irregular morphology. Also, it would be interesting to confirm the ingestion of *E. coli* by protozoa; some studies were performed applying fluorescence to *E. coli* cells or particles to observe if protozoa are feeding on them (De Kreuk, et al., 2010, Li, et al., 2013, Van der Drift, et al., 1977).

In the following paragraphs, the possible causes for the reduction of the ciliates population density observed in the research will be discussed.

Factors affecting ciliates population density

Around day 86, unusual copper precipitation was observed in the influent tubes of some reactors in the laboratory, this led to a general investigation of the copper source. It was found that the tap water contained 0.2 mg/L of copper and the influent water to the reactor had 1.2 mg/L, which is higher than usual. Analysis of the granules of one AGS reactor at TU Delft were performed under SEM and TEM, and they showed a significant amount of copper 'bulbs'. Finally, the source of copper was attributed to the floater system of the tap water supply, which was changed but still contains copper. However, before confirming the source, a general measure of changing tap water to demi-water was applied in all AGS reactors from the laboratory. From this day onwards, copper precipitation was not observed anymore in the tubes. According to Pauli, et al. (2001), concentrations of 1 mg/L of copper might affect the population density of ciliates. Therefore, the high copper concentration in the reactor might have been the cause of protozoa inhibition, and thus, of *E. coli* removal reduction on day 97.

As it can be observed in Graph 4-9, immediately after changing from tap water to demi-water, there was no more removal of *E. coli*. Therefore, at first it was thought that the change from tap to demi-water might have inhibited the protozoa, but conductivity in both types of influent was measured and no significant differences were found (2,070 µS/cm for influent with tap water and 1,885 µS/cm for influent only with demi-water).

Another fact that is important to mention is that on day 93, the DO probe was exchanged with one from another reactor. Despite it was calibrated as usual, it might be that the DO concentration being measured was not correct, i.e. maybe the actual DO concentration was lower than the one measured. Furthermore, two days before day 97, DO was reduced from 50% to 20%, therefore, insufficient oxygen might have inhibited protozoa as well.

Looking at Graph 4-6, it can be observed that on days 82 and 89, VSS and TSS in the reactor reached their maximum value. Sudo and Aiba (1973) demonstrated that despite bacteria limit the growth of protozoa, there is a maximum concentration of bacteria, that if exceeded, the growth rate of protozoa is diminished. Therefore, the high concentration of VSS in the reactor on days 82 and 89 might have affected the consequent growth of ciliates.

E. coli was the only particulate substrate fed into the reactor, but as it is going to be demonstrated in the following sentences, the density of *E. coli* cells was not enough for

feeding all estimated *Vorticella* present. *Vorticella* were not quantified in this research, therefore, as it will be explained further in this chapter, a population density of ciliates in the reactor of 9.6 x 10^3 ind/mL was assumed based on calculations. According to Pauli, et al. (2001), a density of *Vorticella microstoma* of 1 ind/mL, consumes more than 2.8 x 10^3 bacteria/mL/h. Thus, the estimated population will consume 2.7 x 10^7 bacteria/mL/h. The influent to the reactor consisted of 1,500 mL which contained 1 x 10^5 CFU/mL of *E. coli*, the reactor volume was 2.9 L, and the aeration period in which protozoa were active lasted 1.83 hours. In this way, the available *E. coli* for ciliates consumption during aeration was 2.8 x 10^4 CFU/mL/h according to the formula presented in Equation 12, which is far below the required amount for ciliates feeding of 2.7 x 10^7 bacteria/mL/h. As influent *E. coli* bacteria was not enough for growing the protozoa present, it can be stated that there was another source of free-suspended bacteria or particles of the size edible by protozoa (0.3 to 5 µm). This statement is supported by Curds and Fey (1969), who enunciate that if *E. coli* is the only food source, *Vorticella microstoma* survives only for short periods of time. Furthermore, they state that when apart of *E. coli*, other bacteria are available to be eaten by ciliates, *E. coli* are also suitable food for ciliates.

$$Bacteria\ available\ = \frac{10^5 CFU/mL \times 1500\ mL}{2900\ mL \times 1.83\ h} = 2.8 \times 10^4 \frac{CFU/mL}{h} \qquad \textit{(Equation 12)}$$

Therefore, another hypothesis for protozoa inhibition would be that for some reason, in the periods when protozoa activity dropped, bacteria or solids in the size of 0.3 and 5 µm were not sufficient to feed the population of ciliates. According to De Kreuk, et al. (2010), more bacteria are eroded from granules when there is faster growth rate of organisms. The biomass growth yield (Y) was calculated for each sampling period based on the following formula (Metcalf, 2003).

$$Y\ (gCOD/gCOD) = \frac{g\ biomass\ produced}{g\ substrate\ consumed} \qquad \textit{Equation 13}$$

The produced biomass was calculated based on the difference of grams of VSS between two sampling days. The grams of VSS in the reactor for both days and the grams of VSS which went out of the reactor in the sampling period within the effluent and in the samples were taken into account. The grams of VSS were multiplied by the ratio COD to VSS of sludge fcv = 1.48 g COD/g VSS (Henze, et al., 2008). The grams of COD consumed for the sampling period were calculated as the grams of COD in the influent per cycle (considering 0.366 g/L of COD in the influent, and 1.5 L of influent volume), multiplied by the number of cycles per day (8 cycles per day), and by the days of the sampling period. The following formula was used.

$$Y\ (gCOD/gCOD) = \frac{(g\ VSSf - g\ VSSi) \times 1.48}{0.366 \times 1.5 \times 8 \times days\ sampling\ period}$$ *Equation 14*

Between days 27 and 89, the biomass growth yield had been oscillating between 0.22 and 0.55 g COD/g COD, with an average of 0.37 g COD/g COD, which coincides with the standard biomass growth yield for biological phosphate removing AGS systems grown on acetate (De Kreuk, et al., 2010). On day 97, when *E. coli* removal was absent, the biomass growth yield dropped to 0.03 g COD/g COD. After this day, it increased again for the rest of the research period to an average of 0.35 g COD/g COD. This is in line with the stated hypothesis, and therefore one of the causes of the absence of *E. coli* removal on day 97 might have been the lack of food for ciliates provoked by a drop in the growth rate of biomass, eroding less bacteria from granules. Nevertheless, this hypothesis could not be strictly confirmed because particles diameter was not measured.

As the biomass growth yield on days with significant *E. coli* removal coincides with the standard for this kind of systems, it seems that the amount of bacteria eroded from granules in this system should also coincide with the standard, and therefore the amount of ciliates found in this system would be the usual amount for this kind of systems. Although there are studies which confirm that ciliates attach to the granules of AGS systems (De Kreuk, et al., 2010, Lemaire, et al., 2008, Pronk, et al., 2015), and there is one study which quantified *Vorticella* for an AGS reactor fed with raw wastewater (Li, et al., 2013), no quantification of this stalked protozoa in an AGS system with the characteristics of this one could be found. It is recommended for further studies, to repeat these experiments, adding as routine analysis the quantification of ciliates, and the morphology of the granules by means of image analysis as used in Beun, et al. (2002). Also, effluent samples could be observed under the microscope to search for single bacterial cells surrounded by EPS; according to Pronk, et al. (2014), this is a sign of high erosion rates of granules, and therefore, granules instability.

Summarizing, the inhibition of ciliates which provoked reduction of *E. coli* removal might have occurred due to the effects of high copper concentration, lack of oxygen, high VSS, low biomass growth yield, or a combination of them. Further research should be done to elucidate which are the real causes.

Effect of ciliates presence on the performance of the reactor

According to De Kreuk, et al. (2010), protozoa presence in AGS systems lead to less effluent suspended solids. This trend could not be observed in this research comparing Graph 4-9 with Graph 4-7. This is because the TSS in the reactor never reached a stable value, therefore TSS in the effluent was fluctuating for other reasons apart from protozoa presence. However, as stated in Section 2.6, Li, et al. (2013) found out that when protozoa were absent from an AGS reactor, effluent TSS was 103 mg/L, and this value was reduced to 47 mg/L for a *Vorticella* population of 2.4 x 10^4 ind/mL. Assuming a logarithmic correlation between *Vorticella* population and effluent TSS, the corresponding effluent TSS for a *Vorticella* population density

of 9.62 x 10^3 ind/mL is 50 mg/L, which is not far from the average of 69 mg/L obtained in this research.

Pauli, et al. (2001) state that a correlation between effluent COD and the population density of typical ciliates from AS was found in municipal wastewater treatment plants; the higher density, the lower effluent COD. Nonetheless, taking into account COD removal efficiencies of Graph 4-1, COD effluent concentrations of Graph 4-2, and qualitative protozoa activity from Graph 4-9, this correlation could not be observed in this laboratory research for AGS. It is recommended for further studies, to weekly quantify the population density of protozoa to accurately determine the correlations between the population density and other parameters, such as COD.

In Graph 4-9 it can be observed that on day 68, *E. coli* removal is the highest in the entire research period while ammonium removal coincidently decreased. Also, after day 106, when *E. coli* removal starts to increase, ammonium removal is affected. Despite according to Lee and Welander (1994), rotifers and nematodes negatively affect nitrification in aerobic processes taking place in biofilms, Pauli, et al. (2001) state that ciliates presence shows no effect on nitrification. However, Lemaire, et al. (2008) state that stalked ciliates attached to the surface of the granules of a lab-scale AGS reactor fed with abattoir wastewater, generated localized zones of oxygen depletion on the surface of the granule, and this might provoke an oxygen diffusion limitation. Therefore, on day 68, nitrification might have been affected due to the abundance of stalked ciliates.

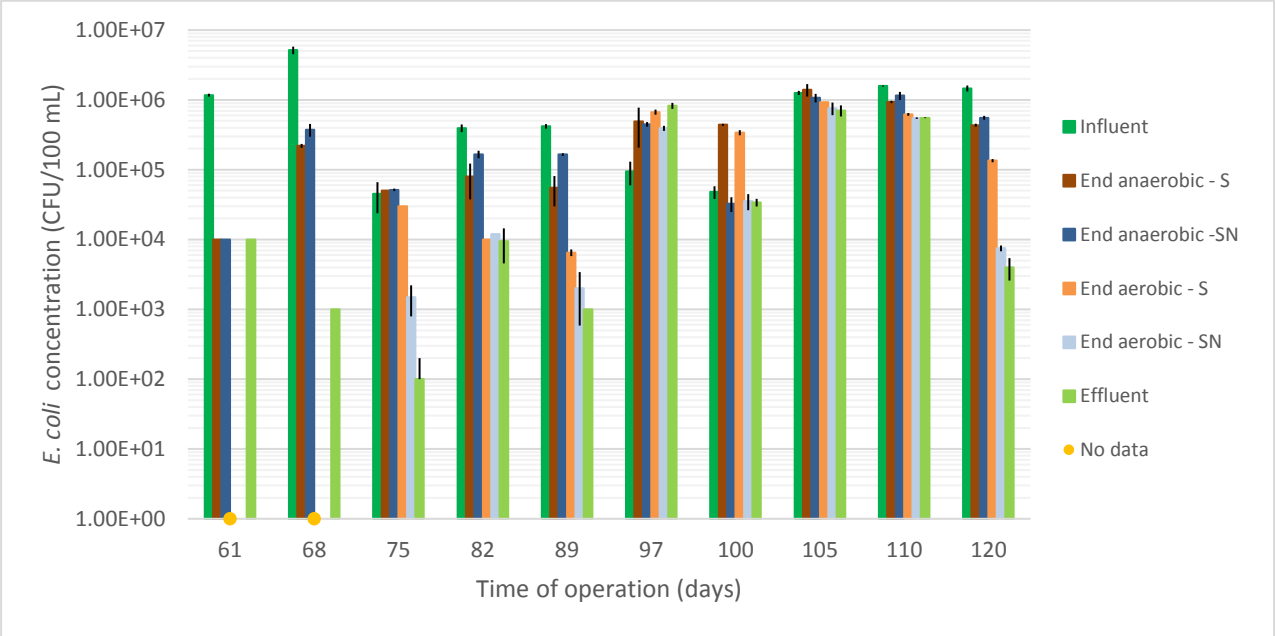
Pauli, et al. (2001) remarked that in full-scale submerged fixed-bed filters, protozoa get attached to the bacterial agglomerations encountering there a favouring environment with plenty of food and appropriate oxygen concentrations. Under these conditions, the proportion of protozoa with respect to the total microbiological population is double the proportion of bacterial biomass, whereas for AS, bacterial biomass is higher in number than protozoa. Although this appreciation was made on the number of individuals, the mass of these two microorganisms will be estimated in the following sentences. Assuming a dry mass of 3.85 x 10^{-6} mg/ind for an individual of *Vorticella microstoma* (Sudo and Aiba, 1973), and a population density of *Vorticella* of 9.6 x 10^3 ind/mL, the resulting mass of *Vorticella* per litre is 0.036 g/L. Comparing this number with the average VSS of 5.8 g/L, which mostly represent bacteria, the proportion of *Vorticella* with respect to the total is far lower than the proportion of bacteria. However, as *Vorticella* were not accurately quantified, it would be interesting for further studies, to measure it and compare the amount of protozoa growing in this AGS reactor with the amount of bacterial biomass.

Fate of *E. coli*

Until now, only the predation of ciliates was indicated as removal mechanism of *E. coli*, but as mentioned in Chapter 2, attachment to bacterial agglomeration is also a removal mechanism, and this will be discussed in this section while studying the fate of *E. coli*.

The following graph shows the fate of *E. coli* for the different sampling days, indicating the concentration of *E. coli* for influent, effluent, sludge and supernatant fractions of end anaerobic, and sludge and supernatant fractions of end aerobic. These results are also

presented in Annex 7. The standard deviation of each measurement, and the missing data, are also indicated.



Graph 4-10: Fate of E. coli AGS (S: Sludge; SN: Supernatant)

As a complementary result to this graph, the concentrations of *E. coli* in influent, effluent, sludge and supernatant at end anaerobic were measured before the addition of *E. coli* to the system. It was found that there was no growth of *E. coli* in the plates, this means that the concentrations were below the detection limit of 10^3 CFU/100 mL.

In general, a volume of 0.1 mL of sample was used for the spread-plate technique in Chromocult coliform agar; as mentioned before, for undiluted samples, this has a detection limit of 10^3 CFU/100 mL. In the case of day 75, the volume of effluent sample plated was increased to 1 mL, in this sense, the detection limit in this case was 10^2 CFU/100 mL.

Different results can be observed in the graph above for the various sampling days. Comparing this graph with Graph 4-9, it can be observed that on days 75, 89, and 120, when *E. coli* removal was significant, a trend can be found, this will be explained in the following paragraphs. Days 82 and 110 also show this trend with slight deviations.

First, *E. coli* concentration at end anaerobic in sludge and supernatant are lower than the influent, being the concentration in the sludge lower than in the supernatant (except for day 75). The reduction of *E. coli* concentration during the anaerobic phase cannot be entirely attributed to the dilution of the influent in the working volume of the reactor as it will be explained in the following sentences. For example, on day 120, as the concentration in the sludge is lower than in supernatant, it can be stated that there is not an equal distribution of *E. coli* in the working volume of the reactor, there are more *E. coli* in supernatant than in sludge. If there was an equal distribution, sludge and supernatant would have the same *E. coli* concentration, therefore sludge would have a higher concentration than the one showed in the graph, and vice versa for the supernatant. If there was an equal distribution, as the

working volume is 2,900 mL, the influent volume is 1,500 mL, and the influent *E. coli* concentration is 1.47 x 10^6 CFU/100 mL, the concentration in the reactor would be 7.6 x 10^5 CFU/100 mL. The measured concentration in the supernatant should be higher than this number, as in reality the supernatant is more concentrated than the sludge, but it is not, the concentration in the supernatant is 5.55 x 10^5 CFU/100mL. Therefore, the reduction of *E. coli* concentration in sludge and supernatant during the anaerobic phase is not only due to dilution, it might also be attributed to natural die-off of some *E. coli* during the anaerobic phase.

Based on the technique used for the separation of sludge and supernatant, it cannot be stated if the *E. coli* present in the fraction sludge are attached to the granules or just free-suspended between them. In further studies, this can be determined by means of centrifugation as in Van der Drift, et al. (1977), where it is stated that free bacteria were not in the sludge pellet after centrifugation. Furthermore, they diluted the samples in sterile solutions of sodium chloride with sodium pyrophosphate and Lubrol W, in order to disperse the *E. coli* attached to particles. This method could be applied in further studies to observe if there are differences with the method employed in this research.

At the end of aerobic, the *E. coli* concentrations in the sludge and supernatant are lower than at the end anaerobic. The reduction in the supernatant can be attributed to protozoa grazing on the free-suspended *E. coli* in the supernatant. Regarding the reduction in the sludge fraction, if all *E. coli* cells were free-suspended, *E. coli* in sludge fraction would always be equal or lower than in supernatant. However, at end aerobic, the sludge concentration is higher than the supernatant. Therefore, it can be assumed that some of the *E. coli* in the sludge at end aerobic are attached in the granules, not being available for protozoa. As mentioned before, this hypothesis cannot be confirmed because the technique used for the separation of sludge and supernatant did not permit to differentiate between physically attached *E. coli* to the sludge, and *E. coli* just dispersed in the fraction.

As mentioned before, at the end aerobic, which is almost the end of the cycle, the concentration of *E. coli* in the sludge is higher than in the supernatant. In fact, the average concentration in the supernatant for days 75, 89, and 120 is 94% lower than in the sludge. The concentration in the sludge represents on average 26% of the influent, and the concentration in the supernatant or effluent represents 0.2% of the influent concentration.

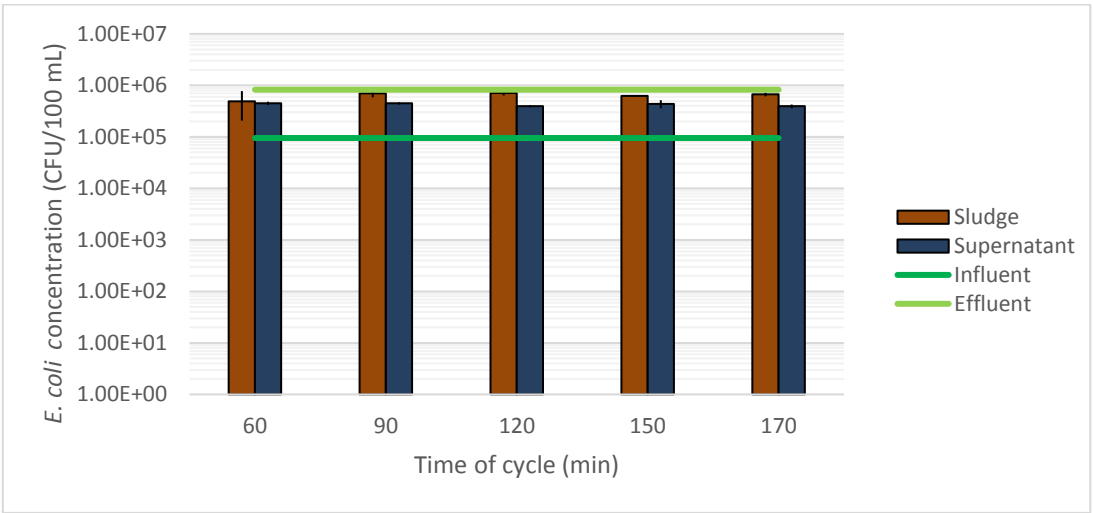
It can also be observed that effluent concentration is always slightly lower than supernatant at end aerobic. Actually, effluent concentration should be almost the same as supernatant at end aerobic, but since the settling in the reactor is more efficient than the settling in the sampling cups, the supernatant at end aerobic has more solids than the real effluent, and as mentioned before, the sludge at end aerobic has more concentration of *E. coli* than the supernatant.

On days 97, 100, and 105 *E. coli* removal was not significant, and a trend in the fate of *E. coli* could not be found. Day 97 showed an influent concentration lower than the rest of the sampling points, this together with the fact that there was no *E. coli* removal, was unexpected, and therefore, the sampling was repeated on day 100 to check for analytical problems. Day 100 showed a different result: influent, supernatants, and effluent had the same

concentrations, whereas sludge fractions had one order more. These two results can be explained as follows.

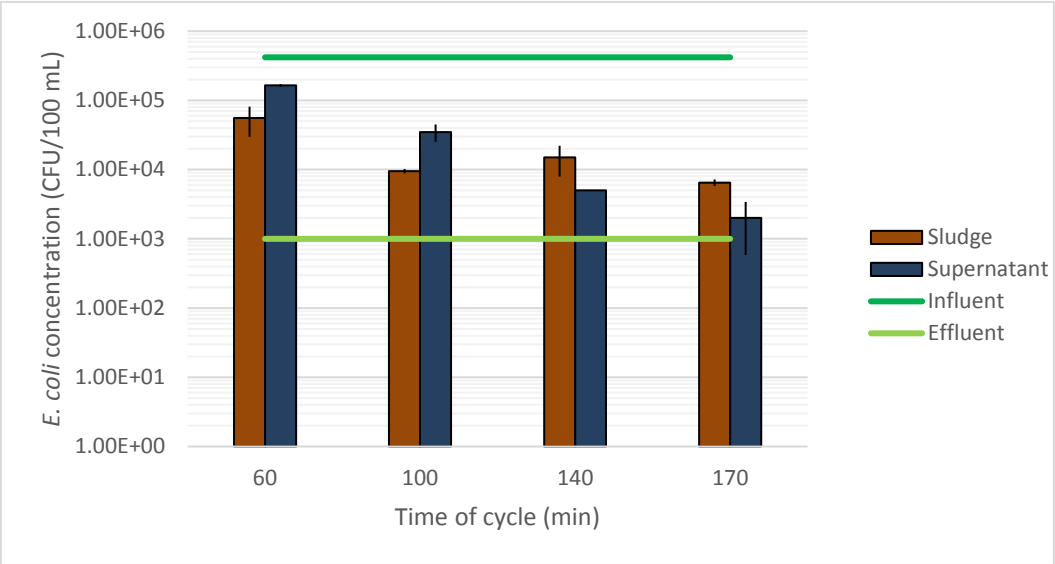
As mentioned before, from day 89 onwards, protozoa were inhibited, i.e. there was no more removal of *E. coli* by protozoa. Day 97 shows a higher concentration of *E. coli* in sludge and supernatant than in influent, this shows an accumulation of *E. coli* in sludge and supernatant from previous cycles. However, as the concentration in the effluent is as high as in the sludge and supernatant, there is no removal. Between day 97 and day 100, protozoa started to slowly be active again, and therefore they started eating free-suspended *E. coli* in the supernatant again, so influent, supernatant and effluent concentrations were equalled. It seems that the *E. coli* cells in the sludge fraction on day 100 were physically attached to the granules explaining the high *E. coli* concentration in this fraction. On day 105, the influent concentration was higher than previously, and it equalled the concentration in the granules. On day 110, the effect of the ciliates starts to be reflected again.

As mentioned in Chapter 3, apart from the samples taken at the end of anaerobic and aerobic, some samplings also included samples in between the aeration phase. This was performed in order to understand the removal processes that were taking place during the aeration phase. On day 89, apart from the samples taken in minute 60 of the cycle (end anaerobic) and in minute 170 (end aerobic) of the cycle, two samples in between aeration were taken, one at minute 100 of the cycle, and the other one at minute 140. On days 97 and 105, apart from minute 60 and 170, samples at minutes 90, 120, and 150 were also taken. As discussed previously, day 89 shows a significant removal of *E. coli*, whereas days 97 and 105 do not. The following graphs indicate the fate of *E. coli* during aeration by presenting the concentration of *E. coli* in the fraction sludge and in the fraction supernatant, for each sample of the aeration phase for days 97 and 89; influent and effluent concentrations, and standard deviation of the measurements are also shown.



Graph 4-11: Fate of E. coli during aeration – Day 97

Since on day 97 there is no *E. coli* removal, this graph shows the same concentration of *E. coli* in the fraction sludge for all samples and the same concentration of *E. coli* in the fraction supernatant for all samples.

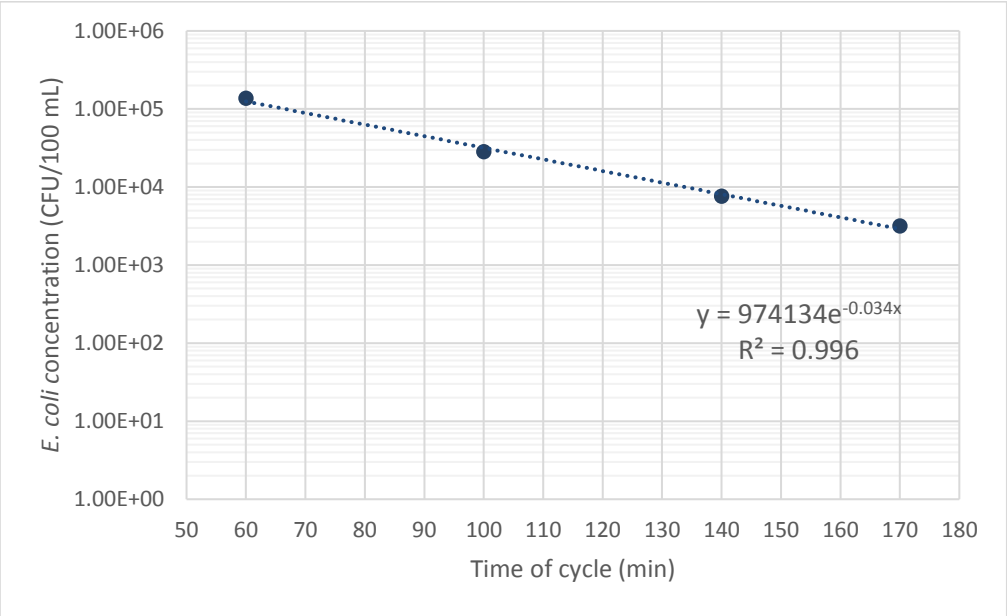


Graph 4-12: Fate of E. coli during aeration – Day 89

In the previous graph, it can be observed again that for day 89, in minute 60 the most concentrated fraction is the supernatant, and in minute 170 the most concentrated fraction is the sludge. Regarding the reduction in the fraction supernatant, as mentioned before, it can be attributed to protozoa grazing on suspended *E. coli* in the supernatant. It seems that the measurement of minute 100 for sludge fraction is mistaken because it is lower than minute 140; if concentration in minute 100 was higher, a constant reduction in the fraction sludge could also be observed, but in a slower pace than for the supernatant.

Logarithmic death of *E. coli* by ciliates predation

Given the fact that the main removal mechanism of *E. coli* in this research was protozoa predation, this process will be discussed in more detail in the following paragraphs. The following graph shows, for day 89, the total concentration of *E. coli* cells in the reactor for the different sampling times. The total concentration of *E. coli* cells was calculated taking into account the concentration in sludge and supernatant, the total volume of the reactor, and the volume of sludge bed of that day, which was 750 mL.



Graph 4-13: Logarithmic death of E. coli AGS – Day 89

The trendline follows a logarithmic death of *E. coli*, as expressed in Section 3.1.5, being the Removal rate constant 0.034 min^{-1}. The half-life of *E. coli* during aeration calculated according to Section 3.1.5 would be 20 min. The Removal rate constant and half-life of *E. coli* was calculated for each sampling day and the results are shown in the following table, indicating also the *E. coli* log removals presented in Graph 4-9. It should be highlighted that this calculation is more accurate for the days when samples between aeration were taken besides end anaerobic and aerobic.

Table 4-1: E. coli Removal rate constant and half-life for different sampling days

Sampling day	*E. coli* Log removal	Removal rate constant (min^{-1})	Half-life (min)
*75	2.7	0.017	41
82	1.6	0.023	30
89	2.6	0.034	20
97	0.0	0.000	> 360
*105	0.3	0.003	231
110	0.4	0.006	116
120	2.5	0.024	29

*Days 61 and 68 are excluded because end aerobic was not sampled

**Day 100 was omitted because *E. coli* concentration was higher at end anaerobic than at end aerobic

Given the table above, it can be stated that the days which have the higher *E. coli* log removal (days 75, 89, and 120), with an average of 2.6 log removal, have the same order of Removal rate constant and Half-life, with averages of 0.025 min^{-1} and 30 minutes respectively. Despite day 82 has a lower log removal, the Removal rate constant is similar to the ones where log removal is higher, because the removal in the fraction sludge was higher than usual.

As mentioned in Section 2.5, *Vorticella microstoma* can filtrate 156 nL/h/ciliate (F). According to Equation 7 from Section 3.1.5, the population density (P) of *Vorticella* can be estimated as:

$$P = \frac{k}{F} = \frac{0.025}{156 \times 10^{-6}/60} = 9.62 \times 10^{3}\ ind/mL \qquad \textit{(Equation 15)}$$

Therefore, the maximum population density of *Vorticella* in the research period can be estimated as 9.62 x 10^3 ind/mL. This population density is in the order of the typical population density of *Vorticella* in wastewater of 5.90 x 10^3 ind/mL given by Pauli, et al. (2001). Furthermore, it is only one order lower than the amount of *Vorticella* quantified in an AGS reactor fed with raw wastewater of 2,37 x 10^4 ind/mL (Li, et al., 2013). Although *Vorticella* were not accurately quantified in this research, samples of day 120 (significant *E. coli* removal) observed under the optical microscope, showed approximately 20 clusters of *Vorticella* per sample of granules. Assuming that the volume of the sample was 0.05 mL, and that there were 6 *Vorticella* per cluster, the resulting number of *Vorticella* is 2.4 x 10^3 ind/mL, which is also in the order of the result of Equation 15.

Assuming the population of ciliates calculated of 9.6 x 10^3 ind/mL, ciliates can filtrate almost 3 times the volume of the reactor in the aeration period. The formula used for the calculation of the filtered volume (VF) was the following.

$$VF = 9.6 \times 10^{3} ind/mL \times 2900\ mL \times 156 \times 10^{-6} \frac{mL}{h} \times 1.83\ h = 7{,}948\ mL \qquad \textit{(Equation 16)}$$

However, *E. coli* cells were not entirely removed, this might be due to inefficiencies of the filtration, or it might be that ciliates do not have the capacity of eating so many *E. coli* within the aeration time.

Other experiments

As mentioned in Chapter 3, other experiments besides the routine sampling were performed in order to check and enhance the sampling procedure. The effect of the different durations of the influent sample collection on the *E. coli* concentration was studied. It was concluded that the most convenient duration of the influent collection is 15 minutes. In some specific weeks, a homogenous sample of pottered granules and supernatant was analysed to compare

the *E. coli* concentration of this sample with the respective fractions of pottered granules and supernatant analysed separately. The results showed that the theoretical and the real value of the *E. coli* concentration in the homogenous sample were quite similar. Both studies are presented in Annex 9.

***E. coli* FISH analysis**

FISH analysis targeting *E. coli* was performed on samples taken on the last month of operation of the AGS reactor from end anaerobic and aerobic, for pottered sludge and supernatant; also, a pure culture of *E. coli*, a negative control, and the combination of both, were analysed. The negative control was a sample of the first month of operation of the AGS reactor during aeration before *E. coli* was added to the reactor. The probes used for this were the EC 1531, ECO1167, Gamma 428, and EUB 338, as presented in Chapter 3. The main aim of this analysis was to test the applicability of the probes EC 1531 and ECO1167 to *E. coli* ATCC 25922. In the case that they were applicable, the same probes were going to be used for an analysis of the *E. coli* distribution in a slice of a granule of AGS, but the results showed that the probes were not appropriate for this *E. coli*.

Another aim of the analysis was to observe if fluorescence targeting *E. coli* could be seen inside ciliates, but this could not be achieved because the samples analysed were fixated according to the regular fixation procedure which includes centrifugation (Annex 6), and according to Van der Drift, et al. (1977), ciliates tend to be ruptured by centrifugation. Therefore, if observation of fluorescence targeting *E. coli* inside ciliates is aimed in further FISH analysis, samples should not be centrifuged. Weber, et al. (2007), developed a method to perform simultaneous FISH to bacteria, protozoa, and fungi.

During the FISH analysis, probe EC 1531 did not show a strong signal of fluorescence and probe ECO1167 did not work. The overlaying and the phase contrast pictures obtained for EC 1531, Gamma 428, and EUB 338 are shown in the following pictures. The pictures obtained for each probe are presented in Annex 10.

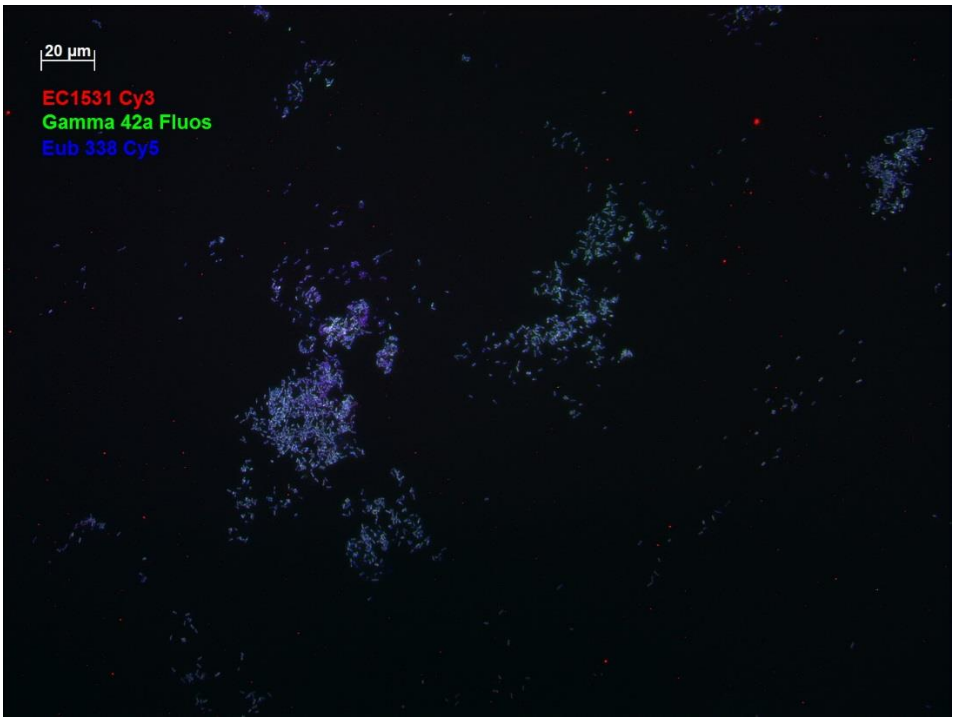


Figure 4-5: E. coli FISH results for AGS – E. coli Pure culture – Overlaying (Red: EC 1531, green: Gamma-protobacteria, blue: All bacteria)

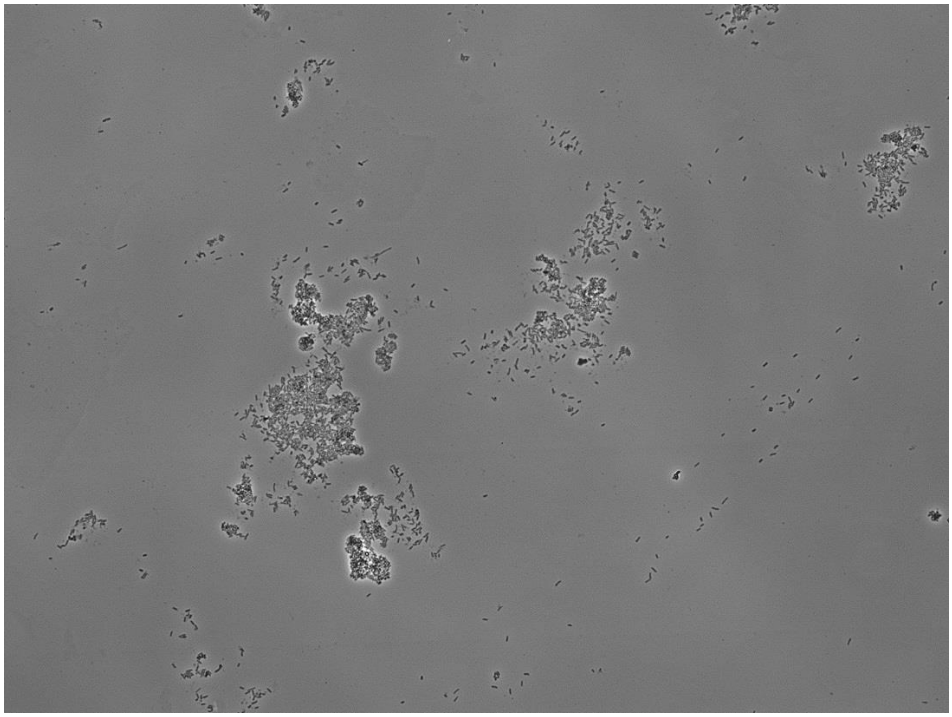


Figure 4-6: E. coli FISH results for AGS – E. coli Pure culture – Phase contrast

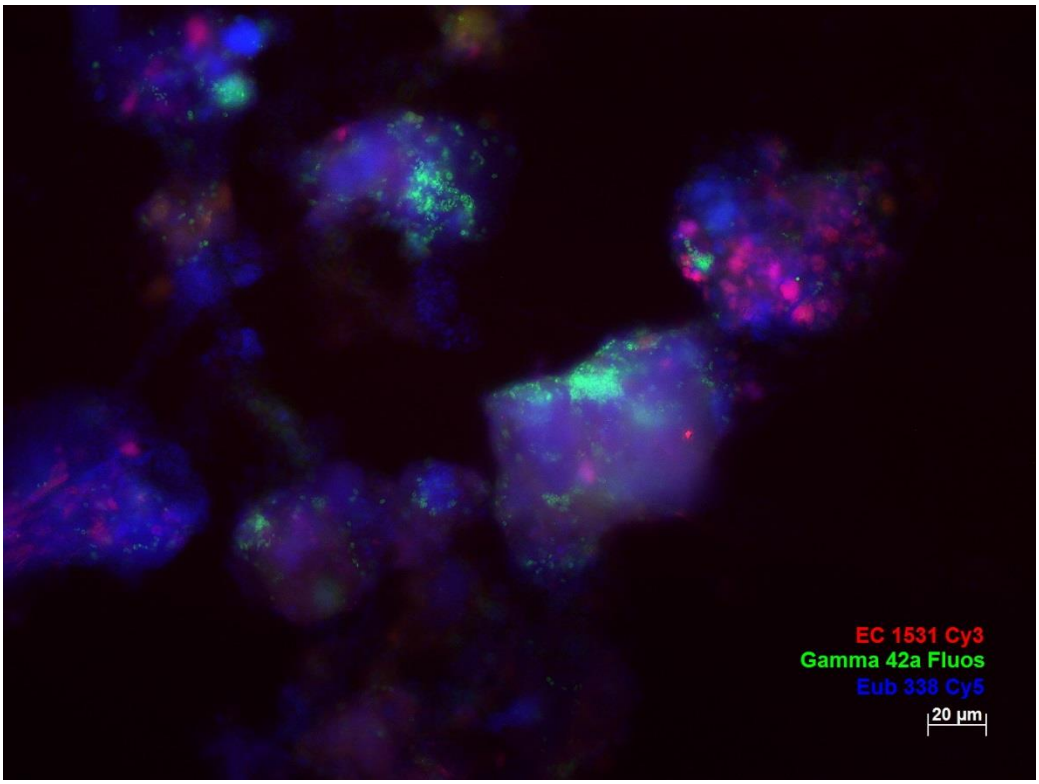


Figure 4-7: E. coli FISH results for AGS - Negative control – Overlaying (Red: EC 1531, green: Gamma-protobacteria, blue: All bacteria)

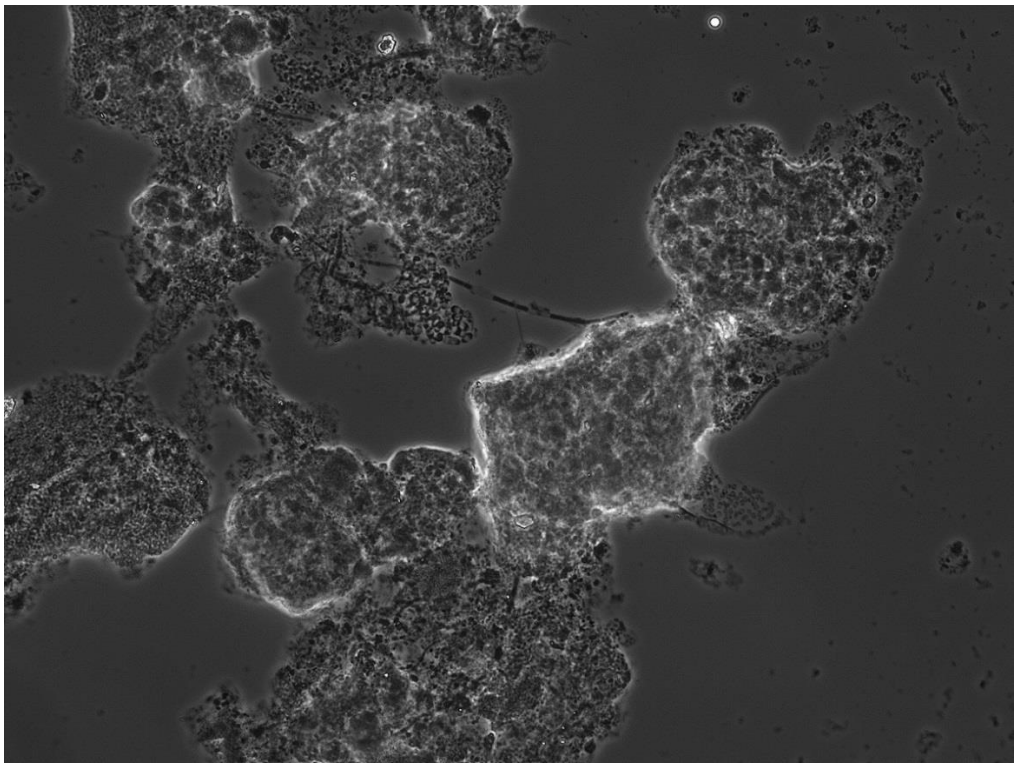


Figure 4-8: E. coli FISH results for AGS - Negative control – Phase contrast

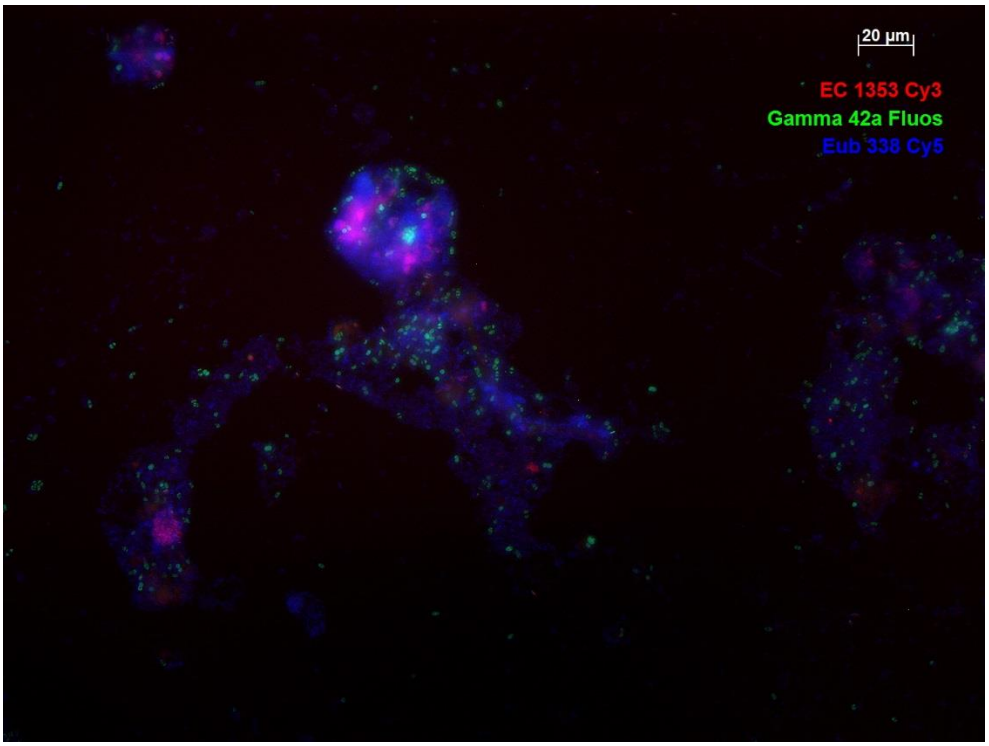


Figure 4-9: E. coli FISH results for AGS - Negative control and pure culture - Overlaying (Red: EC 1531, green: Gamma-protobacteria, blue: All bacteria)

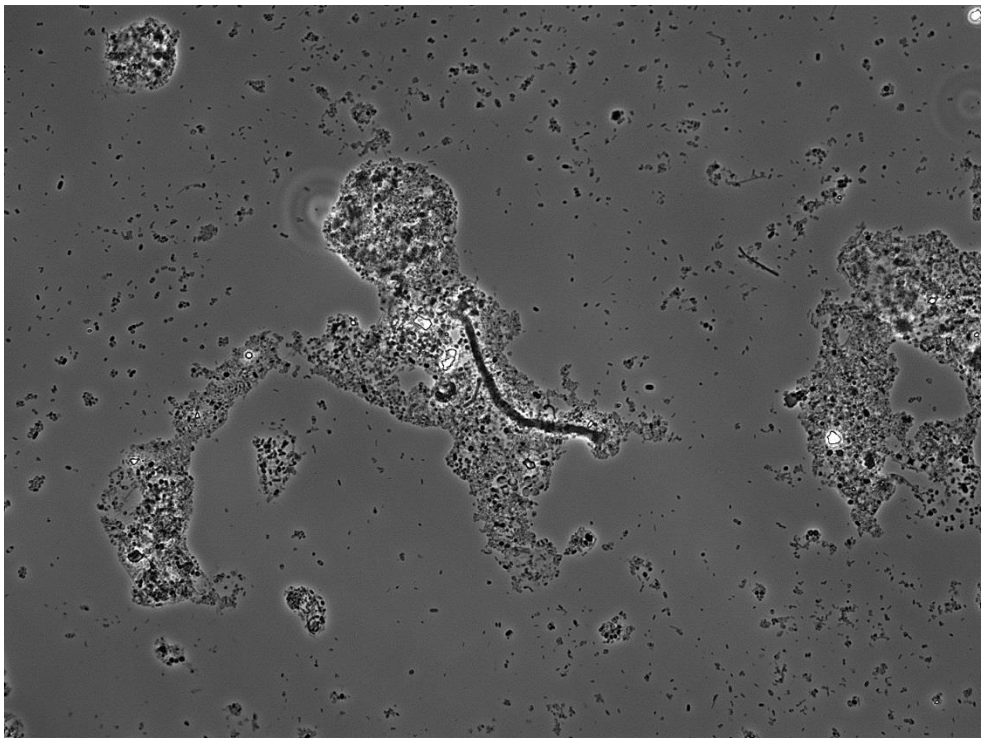


Figure 4-10: E. coli FISH results for AGS - Negative control and pure culture – Phase contrast

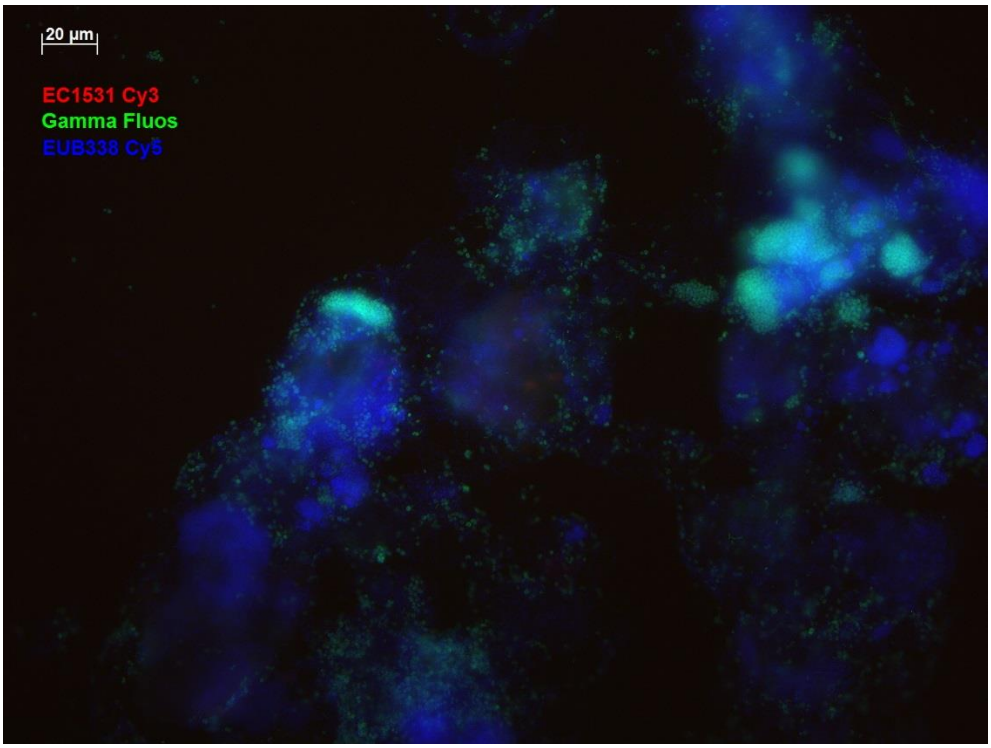


Figure 4-11: E. coli FISH results for AGS - Sample pottered AGS, end anaerobic - Overlaying (Red: EC 1531, green: Gamma-protobacteria, blue: All bacteria)

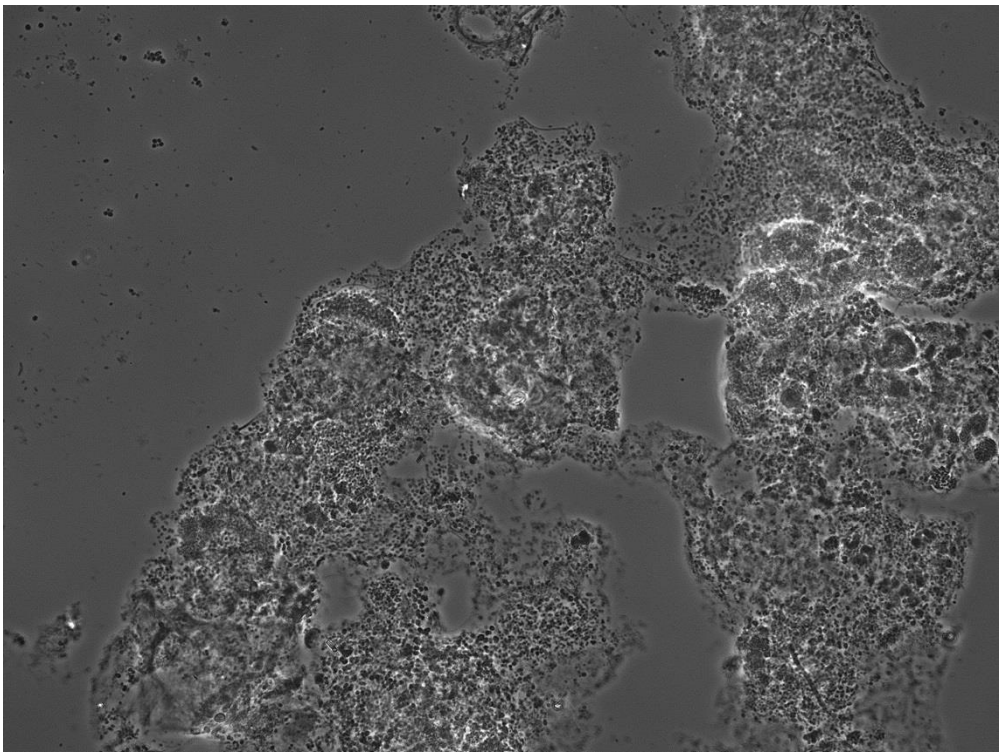


Figure 4-12: E. coli FISH results for AGS - Sample pottered AGS, end anaerobic – Phase contrast

Pure culture:

Figure 4-5 and Figure 4-6 show the results of the FISH analysis for the *E. coli* pure culture. In the figures it can be seen that all the probes overlap. However, while observing under the microscope, it was noticed that some fluorescence was not overlapped, and therefore the culture was not pure, it was contaminated.

Negative control:

Figure 4-7 and Figure 4-8 show the results of the FISH analysis for the *E. coli* negative control. Unexpectedly, the negative control, which is supposed to have just a few amount of *E. coli* or not at all, shows more fluorescence for *E. coli* probes than the pure culture. Furthermore, the *E. coli* probe shows more fluorescent organisms than the Gamma probe, which would not be possible because *E. coli* is a Gamma Proteobacteria. This indicates that the EC 1531 hybridized also with bacteria which are not Gamma-proteobacteria, and thus, it is not specific for *E. coli*.

Pure culture and Negative control:

Figure 4-9 and Figure 4-10 show the results of the FISH analysis for the mixture of pure culture and negative control. These pictures clearly show more organisms fluorescent for the non-specific *E. coli* probes, than for *E. coli* probe. This is logic because the negative control is supposed to have other bacteria than just the ones targeted by EC 1531.

Sample pottered AGS, end anaerobic:

Figure 4-11 and Figure 4-12 show the results of the FISH analysis for the mixture of a sample of pottered AGS at the end anaerobic. These pictures show a very low fluorescence intensity for EC 1531 probe, therefore, the organisms targeted by EC 1531 are not in large quantities during the last month of operation of the AGS reactor.

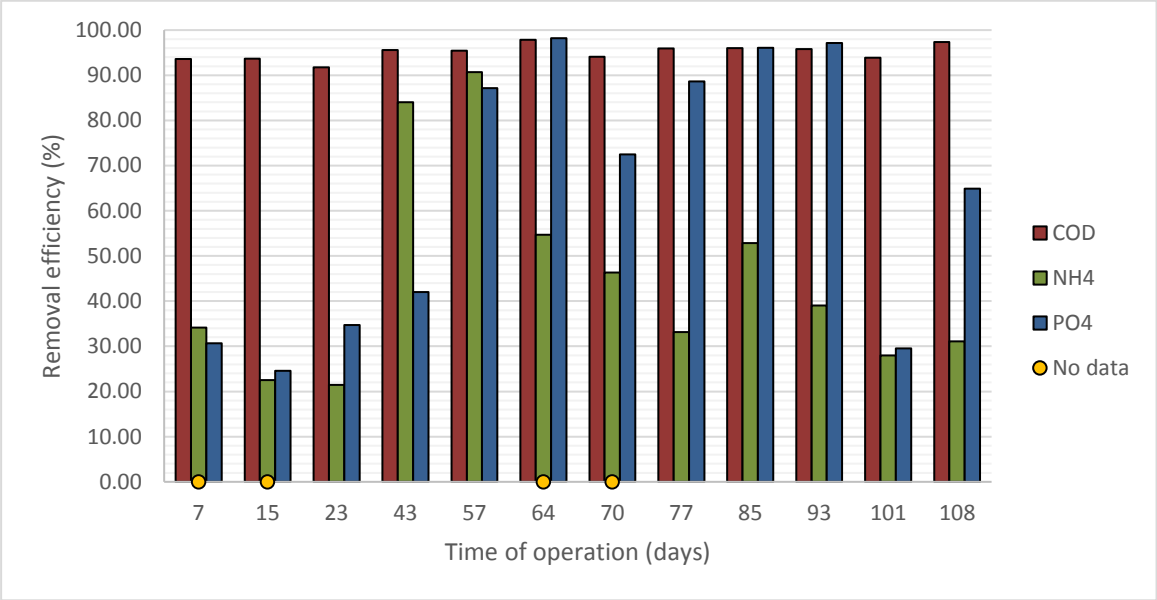
In summary, the most significant finding of this FISH analysis is that neither ECO1167 nor EC 1531 probes are specific for *E. coli*. Therefore, the same analysis to test different probes for *E. coli* was suggested by Mary Luz Barrios and Ben Abbas (TU Delft) to be performed. They designed a specific probe, EC462, for the strain *E. coli* ATCC 25922.

4.2. AS Reactor

4.2.1. *Organic matter and nutrients removal*

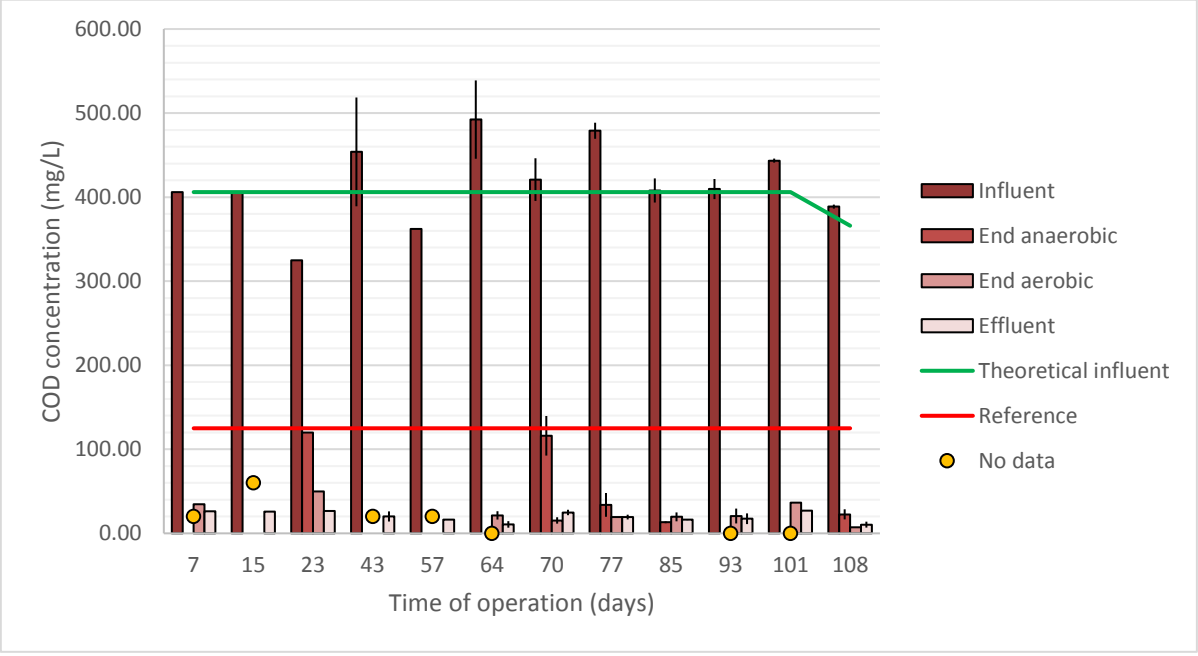
The removal efficiencies for COD, ammonium, and phosphate, and the results for the analyses of influent, end anaerobic, end aerobic, and effluent, all of them for the different days of operation of the reactor, are presented in the following graphs and in Annex 7. The theoretical concentrations of COD, ammonium, and phosphate in the influent is indicated in the graphs. Also, reference values based on the EU standards for the discharges of urban wastewater treatment plants are indicated (Directive, 1991). The missing data is indicated as “No data”; in the case of influent concentrations missing, the theoretical value is indicated instead. For a particular sampling day, if the data of only one sampling point is missing, a value of zero was assigned to the variable “No data”, but when data for more than one sampling point is missing

(e.g. influent and end anaerobic), a value different from zero was assigned to the variable. The standard deviations of the measurements are also shown in the graphs when corresponds.



Graph 4-14: Removal efficiencies of organic matter and nutrients – AS

In the previous graph, it can be observed that the removal efficiency of COD was above 90% during the entire sampling period. Regarding the removal efficiency of ammonium, it is below 60% in almost all cases; on days 43 and 57 it is higher because the reactor was started-up again with fresh biomass. Removal of phosphate is good from day 57 to day 93, oscillating around 90%.



Graph 4-15: Removal of COD – AS

As it can be observed in the previous graph, on the days when end anaerobic was sampled, COD removed is almost all consumed in the anaerobic phase. The effluent is below the reference value for each sampling day. The minimum concentration achieved for COD in the effluent of 10.4 mg/L can be attributed to the same fact as for AGS, according to De Kreuk, et al. (2010), the EDTA contained in the nutrients medium is not biodegradable. Nevertheless, the minimum concentration of COD in the effluent achieved with the AS system is more than 50% lower than for the AGS system, this might be due to the fact that both measurements were performed with different analytical techniques and one might be more accurate than the other one, or due to the fact that there are different organisms living in both systems, so maybe the AS organisms degrade more EDTA than the AGS organisms. In any of these cases, the real average removal efficiency of COD would be around 100%. Looking at day 70, it can be observed that effluent COD is higher than at end aerobic, this might be attributed to an error in the analysis.

Graph 4-16: Removal of ammonia - AS

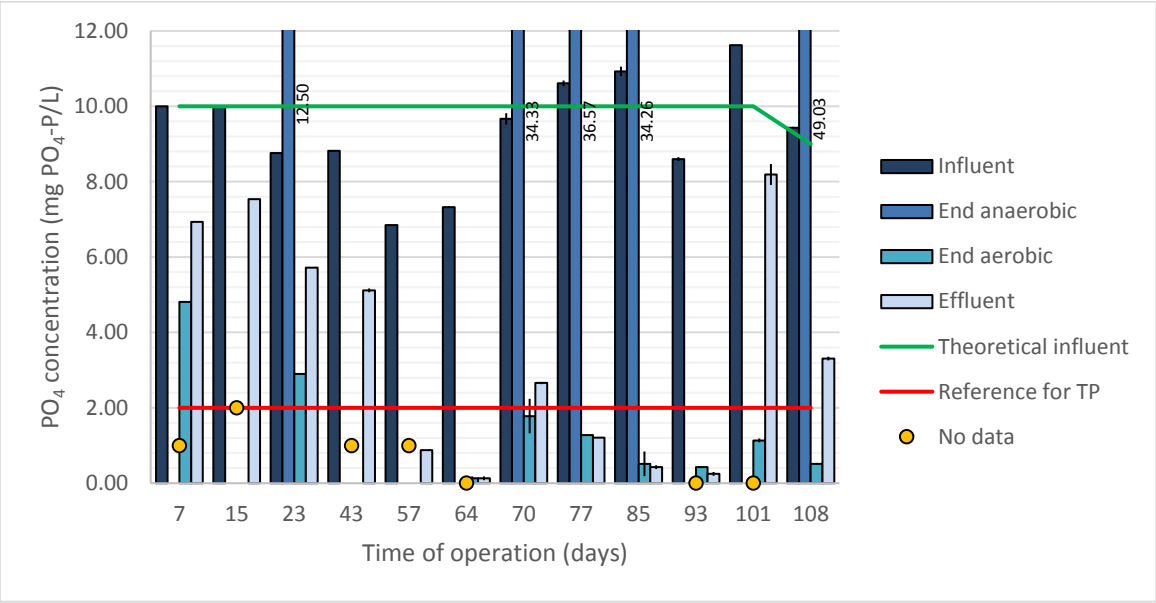
As it can be observed in the previous graph, on the days when end anaerobic was sampled, ammonia removed is almost all consumed in the anaerobic phase. This ammonia reduction might be attributed to the consumption of the biomass for growth, therefore, it can be stated that despite all the modifications introduced in the cycle during the entire research period, nitrification did not take place in the AS reactor. Except for days 43 and 57, none of the sampled effluents comply with the reference value. The low ammonia in the effluent of days 43 and 57 is due to the new start-up of the reactor on day 38 with fresh biomass. During the first month of operation, in the anoxic phase not only COD was fed but also nutrients, this explains a higher ammonia concentration in the effluent than at end aerobic for the first sampling weeks. During the rest of the research period, in some other cases also ammonia

concentration was higher in the effluent than at end aerobic, these can be attributed to analytical errors.

If further studies are to be performed aiming nitrification, it is recommended to start with a cycle based on the cycle of day 85, which showed the best ammonia removal after adaptation of the fresh biomass introduced on day 38. It is also recommended to review the mediums composition, since it is suspected that a component was missing to achieve nitrification in the AS reactor. An example of a medium which worked for nitrification is given by Moussa, et al. (2005), where influent COD was very low to favour nitrifiers growth against heterotrophs, and the initial SRT was of 100 days. The recommended cycle based on day 85 is presented in the following table.

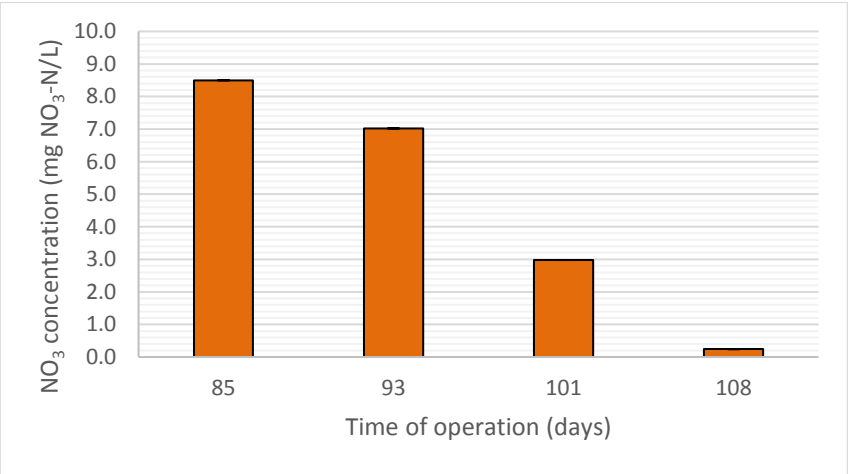
Table 4-2: Recommended cycle for further studies - AS

Phases	Anaerobic					Aerobic	Anoxic			Refresh	WAS	Settling	Effluent discharge	Stand by
Duration (min)	360													
	83					150	46			10	1	39	30	1
	5	3	2	20	53	150	2	22	22	10	1	39	30	1
N_2 gas														
Stirring 500 rpm														
sppH 6.85														
COD 25 mL/min														
Nutrients 25 mL/min														
E. coli 40 mL/min														
Demi-water 160 mL/min														
Compressed air														
spDO 50%														
WAS 0 mL/min														
Effluent discharge														
SRT 30 days manually controlled. HRT 12 hs. pH dead zone 0.3.														



Graph 4-17: Removal of phosphate - AS

From day 57 until day 93 a good biological phosphorus removal can be observed, given the high amount of phosphate at end anaerobic and the low phosphate effluent concentrations, always below the reference value except for day 70. On day 70, as it occurred for ammonia and COD, effluent concentration is higher than end aerobic, therefore, there might have been an error in the sample handling. On days 101 and 108 a release of phosphate can be observed between the end aerobic and the effluent discharge. This is due to two facts, first from day 93 to 101, nitrification was deteriorated as shown in the following graph of nitrate at end aerobic (nitrate generation diminished). Therefore, at the beginning of anoxic phase there were almost no nitrates. Also, between day 93 and 101, in an attempt to achieve nitrification, some phases of the cycle were extended, including the anoxic phase. These two conditions combined, led rapidly to strict anaerobic conditions in the anoxic phase, and therefore a high release of phosphate occurred.

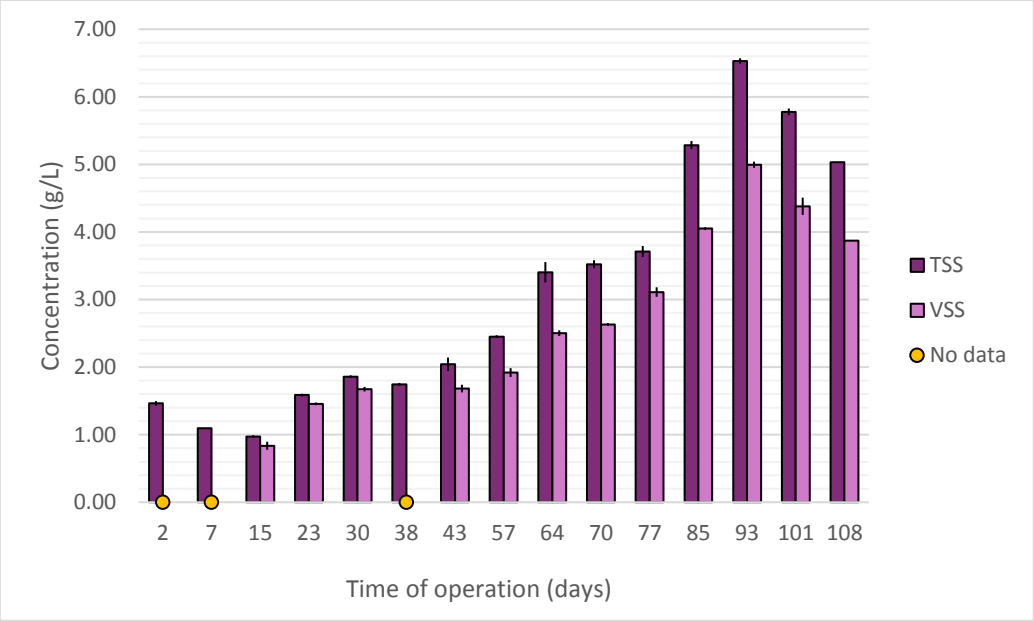


Graph 4-18: Nitrate at end aerobic – AS

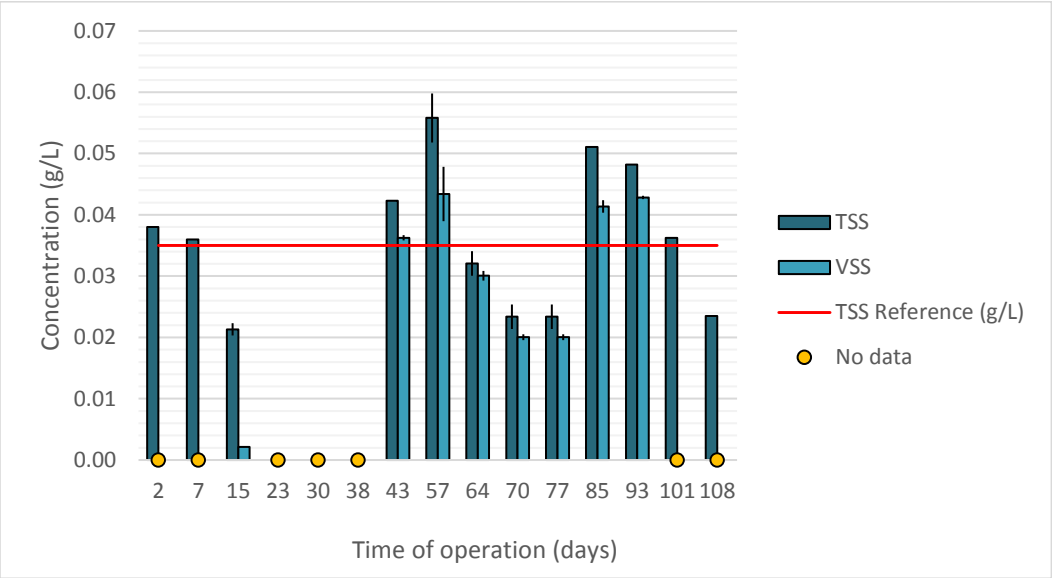
As for AGS samples, FISH analysis was performed to check the presence of PAO's and GAO's in the AS reactor with probes which are proved to work with these organisms. The analysis was done on samples of pottered sludge taken on the last month of operation of the AS reactor during end aerobic. The used probes were PAO651 and GB as presented in Section 3.1.3. Also, DAPI was added. The resulting pictures are presented in Annex 8. The quality of the previous pictures is not very good due to the fact that this analysis was part of a training, and therefore the analysts did not have expertise in hybridization and in acquisition and processing of the images. However, it is possible to identify GAO's and PAO's, and therefore, it can be concluded that both organisms were present in the AS reactor.

4.2.2. *VSS/TSS*

The results for VSS/TSS analyses of the reactor and effluent, and the resulting SRT from performing SRT control, are presented in the following graphs. The VSS/TSS graphs also present the standard deviation of the measurements, reference values based on the EU standards for the discharges of urban wastewater treatment plants (Directive, 1991), and the missing data as "No data". Results for VSS/TSS are also presented in Annex 7. Besides the resulting SRT for each week, SRT graph also shows the desired SRT for each week, and the volume of sludge taken from the reactor each week due to sampling or SRT control.



Graph 4-19: Reactor VSS/TSS – AS

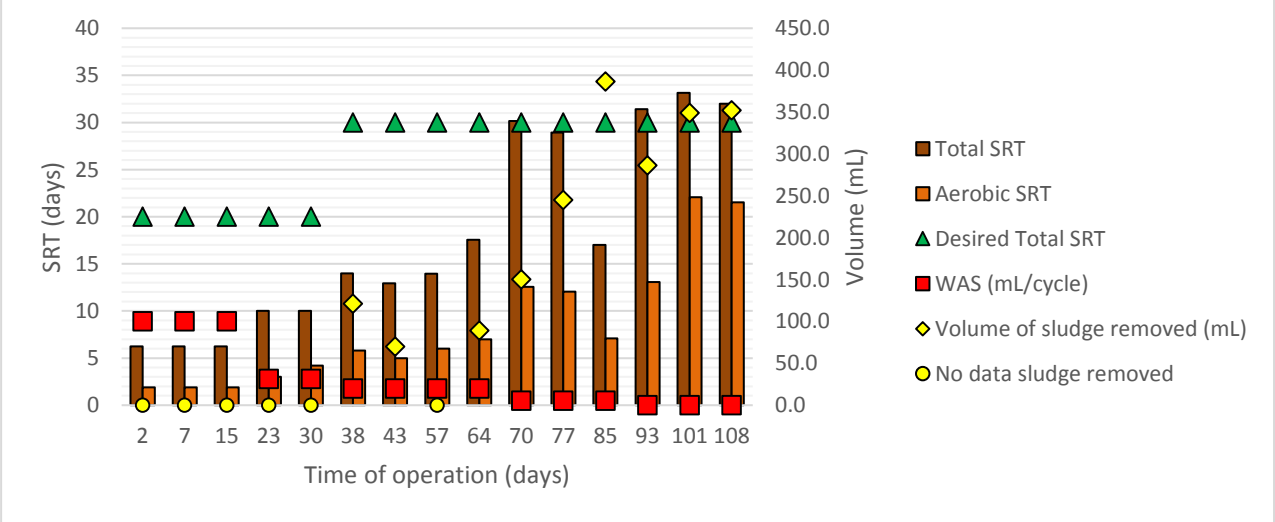


Graph 4-20: Effluent VSS/TSS – AS

As mentioned before, the reactor was started with new biomass on day 38. As it can be observed in Graph 4-19, TSS in the reactor was steadily increasing from this day onwards until reaching 6.5 g/L on day 82, when it started decreasing. During the last two sampling days, TSS decreased until 5 g/L.

Regarding TSS in the effluent, a trend was not found, it fluctuates within the entire research period. Furthermore, values before day 70 are not as accurate as after, because of the same reasons as for the AGS reactor. Taking into account the measurements between day 70 and 108, the average effluent TSS was 0.034 g/L. The reference value is only complied in 40% of the cases.

The average VSS/TSS ratio in the reactor is 81%, and in the effluent it is 86%.



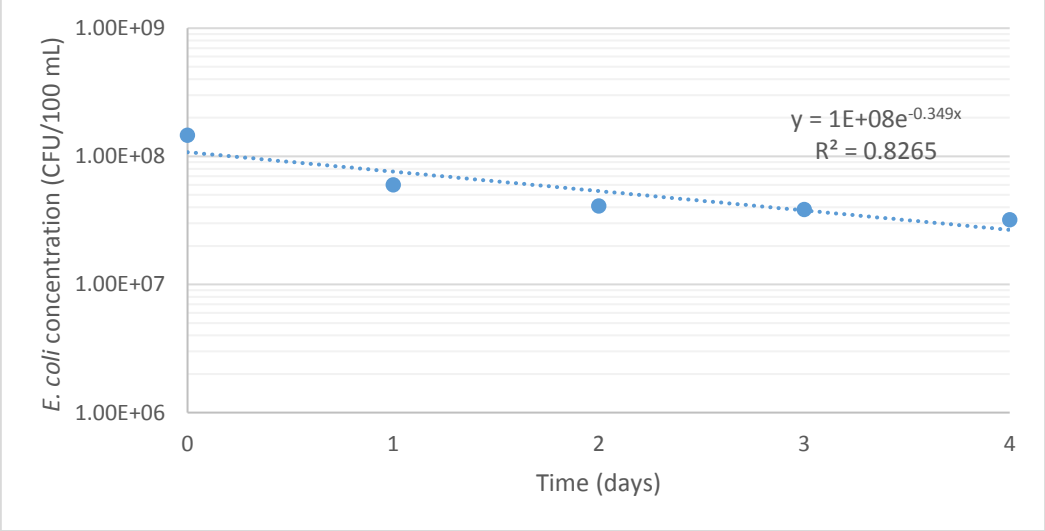
Graph 4-21: SRT – AS

The previous graph shows that the SRT control was only achieved at the end of the research period. Until day 70, desired SRT was not being achieved because of omission of considerations while calculating WAS as it was explained previously in Annex 3. From day 70 onwards, SRT was around 30 days except for day 85. As it can be seen in the same graph, on day 85 a significant volume of samples was taken from the reactor. Until that day, SRT was being controlled by an automatic waste sludge with a WAS pump, but as mentioned before, this modus operando was limiting the volume of samples possible to be taken, since to achieve a given SRT there was a maximum volume of sludge that could be weekly taken from the reactor. On day 85, this maximum was exceeded. And therefore, it was considered more convenient to stop the WAS pump and proceed with manual SRT control as in the AGS reactor. On the last two sampling days, an increased aerobic SRT can be noticed due to the extension of the aeration phase duration between days 93 and 101.

4.2.3. *E. coli removal*

E. coli decay study

At first instance, an *E. coli* decay study was performed between days 78 and 81 with samples from the feeding bottle of the AS reactor. The results are shown in the next graph.

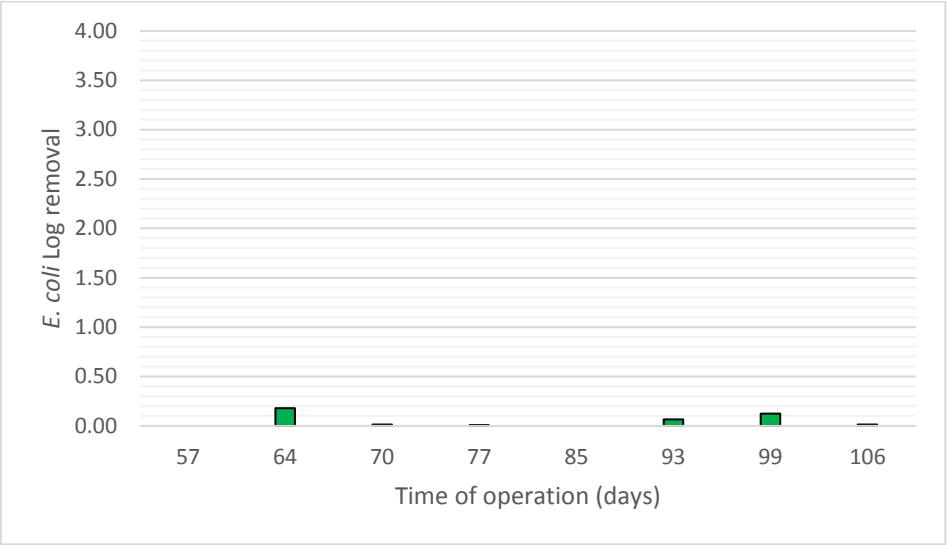


Graph 4-22: E. coli decay in AS feeding bottle, days 78 to 81

This graph shows a logarithmic death of *E. coli* in a 10 L glass stirred bottle, covered with dark plastic, with a Decay rate of 0.349 d^{-1}. Applying the equation of the curve, it can be observed that the concentration of *E. coli* in the bottle would be less than 1 x 10^7 CFU/100 mL after the 7^{th} day of the experiment. Taking into account the dilution of *E. coli* medium with the rest of the mediums, the influent to the reactor would have been approximately 1 x 10^6 CFU/100 mL, which is the minimum concentration desired of *E. coli* in the influent. Therefore, it was concluded that the *E. coli* medium had to be replaced at least every 7 days.

E. coli log removal

The log removal of *E. coli* taking into account influent and effluent *E. coli* concentrations is presented in the next graph and in Annex 7.



Graph 4-23: Log removal of E. coli - AS

In the previous graph, it can be observed that the removal of *E. coli* is not significant on any of the sampling days. The maximum achieved was on day 64 with 0.18 *E. coli* log removal. Days 57 and 85 did not present any removal. Therefore, it can be stated that there was no removal of *E. coli* in the AS system, neither by protozoa predation nor by attachment to the sludge flocs.

Despite the fact that the origin of the seed sludge for the AGS reactor and for the AS reactor was the same, samples of AS reactor observed under the optical microscope on days 70, 85, 91, 92, 93, 99, and 106 did not show any ciliates. In some cases, rotifers were identified, but not in significant amounts, just one in every two samples. The following picture corresponds to an optical microscope snapshot of day 70, showing no ciliates presence.

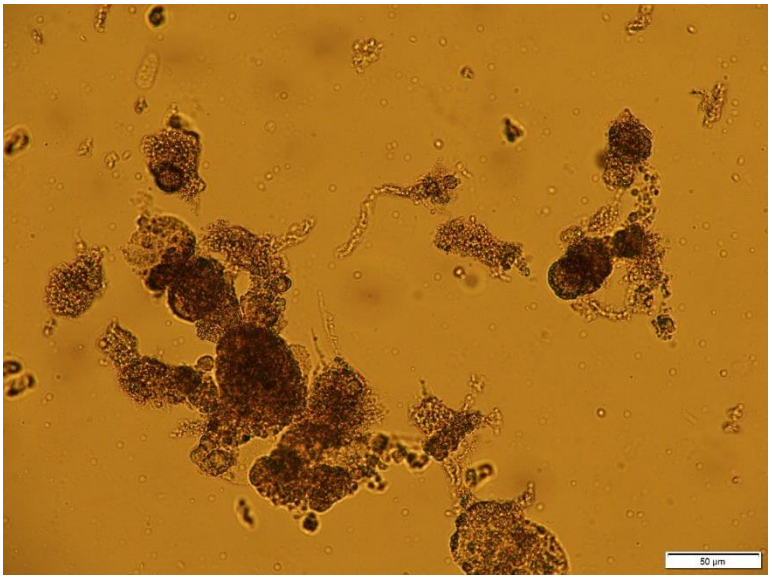


Figure 4-13: Optical microscope snapshot showing no protozoa in sludge of AS reactor – Day 70

Therefore, it can be stated that ciliates which were present in the seed sludge did not survive in the AS reactor, and as ciliates predation is the main removal mechanism of *E. coli* in AS, this fact can justify the absence of *E. coli* removal in this reactor. Different hypothesis were handled as causes of the absence of ciliates in the AS system.

Absence of ciliates

At first it was thought that the long anaerobic phase might have been the cause. However, according to Pauli, et al. (2001), almost all protozoa species in AS seem to survive to low DO concentrations for at least 4 hours (240 minutes). To complement, Van der Drift, et al. (1977) proved that when aerating an AS lab-system previously gassed with nitrogen for 2.5 hours approximately, the same *E. coli* removal curve as without gassing nitrogen, was obtained (attributed to adsorption in flocs and predation by ciliates). However, when aerating after gassing 5 hours with nitrogen, there was no more *E. coli* removal and protozoa were not present anymore. Therefore, as in this case the maximum duration of the anaerobic phase was 135 minutes, which is less than 4 hours, it is considered that the anaerobic phase and its nitrogen gassing did not affect the ciliates population.

As mentioned in Section 2.5, flocs are hiding spots for preys to stay away from their predator. Therefore, agglomeration of bacteria in AS flocs might prevent them from being eaten by protozoa. Initially, it was thought that the protozoa originally present in the seed sludge died because *E. coli* cells fed to the AS reactor were being enmeshed into the floc and due to this, they were not reachable for protozoa. But as mentioned before, there was no removal of *E. coli* by attachment to the flocs, therefore, all *E. coli* were free-suspended in the reactor available for protozoa. However, as demonstrated in Section 4.1.3, the amount of *E. coli* fed in the reactors was not enough to feed the protozoa present in the AGS reactor. In the case of the AGS reactor it was concluded that apart from *E. coli*, there was another source of free-suspended bacteria which was serving as food for the protozoa. According to Macek (1989) the most relevant factor influencing the presence of protozoa in AS is the feeding characteristics of the system, therefore, it can be hypothesised that ciliates did not survive in the AS system because there was not another source of free-suspended bacteria apart from *E. coli*.

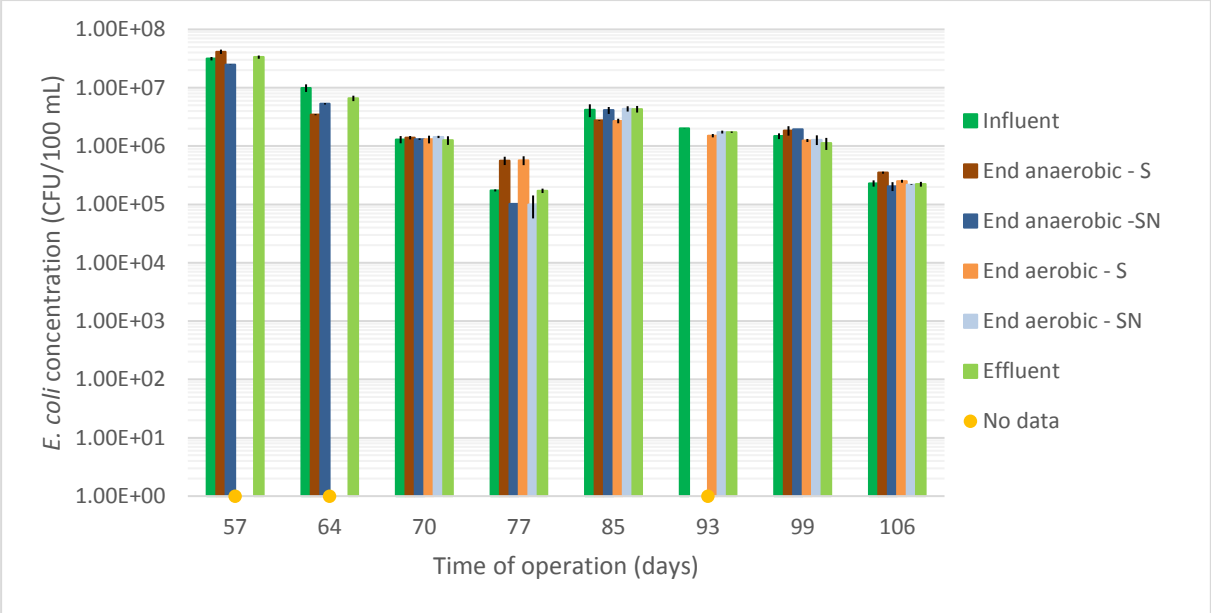
Therefore, it can be stated that in this AS SBR reactor subjected to the cycles mentioned before and fed with the same synthetic wastewater as the AGS system, ciliates cannot survive. If in further studies, the effect of ciliates on AS *E. coli* removal is aimed to be studied, the medium composition should be adjusted in order to favour ciliates presence, an example of a synthetic wastewater feeding an AS system with ciliates is given by Macek (1989).

Absence of attachment to flocs

As mentioned before, one mechanism of *E. coli* removal in AS is the attachment of *E. coli* cells to bacterial flocs, which did not occur in this experimental set-up. According to Loge, et al. (2002), and as mentioned in Section 2.7, with the increase of SRT, the particles associated with coliform bacteria decrease and the free-suspended coliform bacteria increase. Therefore, the high SRT of 30 days might have provoked the non-attachment of the *E. coli* to the flocs.

Fate of *E. coli*

The following graph shows the fate of *E. coli* for the different sampling days, indicating the concentration of *E. coli* for influent, effluent, sludge and supernatant fractions at end anaerobic, and sludge and supernatant fractions at end aerobic. These results are also presented in Annex 7. In this graph, the standard deviation of each measurement, and the missing data, are also indicated.



Graph 4-24: Fate of E. coli - AS

E. coli concentrations in influent, effluent, sludge, and supernatant are all very similar as it can be observed in the graph above, showing no removal of *E. coli* in none of the sampling days. On day 77, it can be observed that the influent is lower than usual and *E. coli* concentration is higher in the sludge fraction than in the rest of the sampling points. Despite there is no removal of *E. coli* due to attachment in flocs, this event might be explained due to some sort of physical attachment of *E. coli* to the sludge flocs during the previous cycles, which maintained the concentration of *E. coli* similar to the influent from day 70. This hypothesis cannot be confirmed because the technique used for the separation of sludge and supernatant did not permit to differentiate between physically attached *E. coli* to the flocs, and *E. coli* just dispersed in the fraction sludge. It can be concluded that the fate of *E. coli* in an AS reactor of these characteristics cannot be studied because of the absence of *E. coli* removal.

4.3. Evaluation of AGS applicability in Uruguay

Garmerwolde, located near Groningen, the Netherlands is a successful full-scale AGS WWTP which complies with the local discharge effluent requirements and achieves the energy savings characteristics of AGS plants. The characteristics of this WWTP were presented in Section 2.1.1.

A comparison between the characterization of the raw wastewater influent to Garmerwolde WWTP (Table 2-3) and the pre-treated wastewater of Montevideo, and the raw wastewater of Mercedes, given in Table 2-7 and Table 2-9 respectively, was done. Moreover, a comparison between the achieved effluent concentrations in Garmerwolde and the Uruguayan discharge standards were done.

For Mercedes, it was observed that the parameters COD, TSS, TN, and TP were less concentrated than for Garmerwolde WWTP. BOD was slightly more concentrated in Mercedes, but the difference of 6% is considered insignificant.

For Montevideo, it was observed that the parameters BOD, COD, TSS, ammonium, and TP were less concentrated than for Garmerwolde WWTP. However, the high conductivity in Montevideo's wastewater is an exceptional characteristic. Data regarding conductivity for Garmerwolde WWTP was not found. As mentioned in Section 2.8.1, average conductivity in Montevideo's wastewater is around 12,000 µS/cm, and in 80% of the measurements (20^{th} percentile), the conductivity was above 3,000 µS/cm.

According to Calver, et al. (2009), when the dominant salt of a saline water is sodium chloride, the conversion of conductivity to salinity can be performed taking into account that 1 µS/cm is equivalent to 0.64 mg/L Cl^-. Therefore, assuming that Montevideo's wastewater's high conductivity is attributed to intrusion of saline water, the average conductivity would be equivalent to an average salinity of 7.68 g/L Cl^-, the 20^{th} percentile would be 1.92 g/L Cl^-, and the 80^{th} percentile according to Graph 2-1, would be around 12.2 g/L Cl^-.

As mentioned in Section 2.9, the results of an AGS SBR fed with synthetic wastewater containing different salt concentrations showed that: ammonium removal was not affected by any salt concentration, granules size and denitrification only decreased for 20 g/L Cl^-, and nitrite oxidation and phosphate removal decreased for concentrations higher than 13 g/L Cl^-. As the 80^{th} percentile of salinity in Montevideo's wastewater is around 12.2 g/L Cl^-, it seems that neither the granules size nor the biological removal of nitrogen and phosphorus in and AGS system would be affected by this wastewater.

Comparing the effluent characteristics of Garmerwolde WWTP presented in Table 2-3 and the Uruguayan discharge standards given in Table 2-6, it can be observed that the concentrations of the parameters given for Garmerwolde effluent comply with the Uruguayan standards (Faecal coliforms, pH, and fats and oil concentrations of Garmerwolde effluent were not found in literature).

Based on the basic data obtained about Mercedes' wastewater, it seems that this wastewater has no exceptional characteristic which could hamper the good performance of an AGS WWTP designed specifically for its characterization and flows to comply with the local standards.

Therefore, it seems that AGS treatment technology would be applicable for Mercedes' wastewater. Furthermore, taking into account the footprint and energy savings that this technology offers, the resulting AGS plant could be more cost-effective than a conventional system, making feasible its funding and construction. To confirm this statement, further detailed studies and a pre-design of the plant should be done taking into account a detailed characterization of the raw wastewater, dry and wet weather flows, costs of civil works, equipment, labour, energy, land, maintenance costs, etc. Also, it should be taken into account that the personnel should be specifically trained to operate the system.

In the case of Montevideo, it also seems that the treatment of its wastewater with AGS technology would be advantageous, providing a cost-effective solution which is capable of complying with the standards. However, this statement would be only confirmed after a full-scale AGS system subjected to saline wastewaters, or a lab-scale AGS system subjected to Montevideo's real wastewater, is proven to be successful.

CHAPTER 5

Conclusions and recommendations

5.1. Conclusions

It can be concluded that an AGS laboratory-scale reactor performing organic matter, nitrogen, and phosphate removal, with alternated anaerobic and aerobic phases, started with crushed AGS from a full-scale WWTP, and fed with synthetic wastewater, is able to achieve a maximum *E. coli* log removal of 3.5, measured with the spread-plate technique. The main *E. coli* removal mechanism appears to be predation by ciliates of the genus *Vorticella*. It seems that removal by attachment of *E. coli* cells to the granules also occurs, but in a less significant proportion. However, provided the method applied for the separation of sludge and supernatant, it is not possible to quantify it. Regarding the fate of *E. coli* for the days when *E. coli* removal is significant, on average, 26% of the influent *E. coli* ends-up in the granules, and 0.2% in the effluent.

On the other hand, an AS laboratory-scale reactor performing organic matter and phosphate removal, with alternated anaerobic and aerobic phases, started with AS originated from the same influent wastewater, fed with the same synthetic wastewater, subjected to the same temperature and range of DO concentrations, as for the previously mentioned AGS reactor, does not remove *E. coli*. Ciliates do not survive under these conditions. This was mainly attributed to the lack of particulate food for ciliates, which is presumable present in an AGS reactor due to bacteria eroded from granules.

Further studies are needed to quantify the attachment of *E. coli* to granules, and to accurately determine the correlation between the population density of ciliates present and the *E. coli* removal achieved in an AGS reactor of these characteristics. Moreover, further research should be done to have a better comprehension of the factors affecting the presence of ciliates in an AGS and in an AS reactor of these characteristics.

After performing a basic analysis comparing the characterization of the wastewater of two localities in Uruguay and the Uruguayan standards, with the influent and effluent characteristics of a successful full-scale AGS WWTP, AGS treatment technology seems to be suitable for treating the wastewater of Mercedes and Montevideo aiming to achieve Uruguayan standards. However, further studies should be performed to confirm this appreciation.

5.2. Recommendations for further studies

Some hypothesis made during this research need to be further studied to be confirmed. In this line, it is recommended to run the AGS and the AS reactors one more time, incorporating the suggestions which were already given, and which are summarized as follows.

In order to accurately determine the correlation between the population density of ciliates and the *E. coli* removal in a lab-scale AGS reactor, it is suggested to perform experiments aiming at artificially increasing or reducing the amount of protozoa. The population density of ciliates should be quantified from the beginning of the reactor's operation, for instance with light microscopy or qPCR. Moreover, it would be interesting to confirm the ciliates' ingestion of *E. coli* by applying fluorescence to the cells of this bacteria and microscopically observing if protozoa are feeding on them, as it had been previously reported in literature.

The correlation between some parameters (such as COD, ammonium, suspended solids in the effluent, and proportion of biomass) and the presence of ciliates in AGS and AS systems, had already been reported in literature. It would be interesting to investigate these correlations in the studied system based on the accurate quantification of ciliates, and compare them with the reported correlations.

In order to further investigate the factors which affect the presence of ciliates in the AGS reactor, it is suggested to determine the size of the granules present each week by studying the morphology of them by means of image analysis. Moreover, effluent samples could be observed under the microscope to search for single bacterial cells surrounded by EPS, being this, a sign of high erosion rates of granules.

Aiming to differentiate between free-suspended *E. coli* in the sludge fraction and *E. coli* cells attached to granules, centrifugation of the samples can be applied as reported in literature. Also, the dispersion of the attached *E. coli* by diluting the samples in solutions, as reported in literature, can be applied to compare the results with the findings of this research.

Further studies should be performed to find the appropriate FISH probes to target the *E. coli* strain used for this research. Once the probes are proven to work, FISH analysis aiming at the observation of *E. coli* inside ciliates could be performed following the reported methods for simultaneous FISH for bacteria and protozoa. Furthermore, in order to confirm the presence of *E. coli* in the granules, FISH analysis of slices of granules could also be performed.

Regarding the AS reactor, if further studies are to be performed aiming to achieve nitrification, it is recommended to run the cycle which showed the highest ammonia removal in this research, and modify the mediums' composition based on mediums which are proven to enhance nitrifiers. Furthermore, the mediums should also be modified based on literature if the survival of ciliates in the reactor is aimed.

In order to further study the applicability of AGS for the treatment of Montevideo's saline wastewater, it is recommended to perform AGS lab-studies with this wastewater.

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ANNEXES

Annex 1: AGS and AS mediums

***E. coli* medium**		
Compound	Concentration (mL/L)	
	AS reactor	AGS reactor
E. coli ATCC 25922 10^{11} CFU/100 mL*	0.625	1

COD and nutrients mediums				
Compound	Concentration (g/L)	COD	NH_4	PO_4
COD medium		3660 mg/L		
$C_2H_4O_2Na.3H_2O$	7.785	0.470 g/g		
$MgSO_4*7H_2O$	0.44			
KCl	0.175			
Nutrients medium			600 mg/L	93 mg/L
NH_4Cl	2.289		0.26 g/g	
K_2HPO_4	0.349			0.18 g/g
KH_2PO_4	0.136			0.23 g/g
10 mL/L Trace metal solution				

Trace metal solution	
Compound	Mass (g)
Na_2-EDTA	50
$ZnSO_4·7H_2O$	2.2
$CaCl_2$	5.54
$MnCl_2.4H_2O$	5.06
$FeSO_4·7H_2O$	4.99
$Na_6Mo_7O_{24}·4H_2O$	1.69
$CuSO_4·5H_2O$	1.57
$CoCl_2·6H_2O$	1.61
H_2O	1000

**E. coli* inoculum was prepared adding 1 mL of *E. coli* to 50 mL of sterile Nutrient broth and incubating this for 24 hs in a rotary shaker at 37 °C

Annex 2: Statements for an AS cycle

```
# Initialization (phase 0)
phase=0
cycle=0
spph=7.0
spdo2=NA
spstirr=500
pmpinfl=0
pmpefl=0
n2=0
air=0
was=0
PROCSTEP=0
NEXTTIME=0
NTT=0
# Anaerobic phase
# phase 1.1
IF PROCSTEP=0 AND TIME>=NEXTTIME
cycle=cycle+1
phase=1.1
spph=7.0
spstirr=500
NEXTTIME=NTT+5
NTT=NEXTTIME
PROCSTEP=1
ENDIF
# phase 1.2
IF PROCSTEP=1 AND TIME>=NEXTTIME
phase=1.2
pmpinfl=1
vent=1
NEXTTIME=NTT+3
NTT=NEXTTIME
PROCSTEP=2
ENDIF
# phase 1.3
IF PROCSTEP=2 AND TIME>=NEXTTIME
phase=1.3
vent=0
pmpinfl=1
NEXTTIME=NTT+2
NTT=NEXTTIME
PROCSTEP=3
ENDIF
# phase 1.4
IF PROCSTEP=3 AND TIME>=NEXTTIME
phase=1.4
pmpinfl=0
NEXTTIME=NTT+20
NTT=NEXTTIME
PROCSTEP=4
ENDIF
# phase 1.5
IF PROCSTEP=4 AND TIME>=NEXTTIME
phase=1.5
NEXTTIME=NTT+53
NTT=NEXTTIME
```

```
PROCSTEP=5
ENDIF
# Aerobic phase (phase 2)
IF PROCSTEP=5 AND TIME>=NEXTTIME
phase=2.1
spdo2=50
spstirr=500
NEXTTIME=NTT+150
NTT=NEXTTIME
PROCSTEP=6
ENDIF
# Anoxic phase 1 (phase 2.2)
IF PROCSTEP=6 AND TIME>=NEXTTIME
phase=2.2
spdo2=NA
vent=1
NEXTTIME=NTT+2
NTT=NEXTTIME
PROCSTEP=7
ENDIF
# Anoxic phase 2 (phase 2.3)
IF PROCSTEP=7 AND TIME>=NEXTTIME
phase=2.3
vent=0
NEXTTIME=NTT+22
NTT=NEXTTIME
PROCSTEP=8
ENDIF
# Anoxic phase 3 (phase 2.4)
IF PROCSTEP=8 AND TIME>=NEXTTIME
phase=2.4
n2=0
NEXTTIME=NTT+22
NTT=NEXTTIME
PROCSTEP=9
ENDIF
# Refresh phase (phase 2.5)
IF PROCSTEP=9 AND TIME>=NEXTTIME
phase=2.5
air=1
NEXTTIME=NTT+10
NTT=NEXTTIME
PROCSTEP=10
ENDIF
# WAS phase (phase 3)
IF PROCSTEP=10 AND TIME>=NEXTTIME
phase=3
pmpefl=0
air=0
spdo2=NA
spph=NA
was=1
NEXTTIME=NTT+1
NTT=NEXTTIME
PROCSTEP=11
ENDIF
# Settling phase
# phase 4.1: settling
IF PROCSTEP=11 AND TIME>=NEXTTIME
phase=4.1
pmpefl=0
spstirr=0
```

```
was=0
NEXTTIME=NTT+39
NTT=NEXTTIME
PROCSTEP=12
ENDIF
# phase 4.2: effluent
IF PROCSTEP=12 AND TIME>=NEXTTIME
phase=4.2
pmpefl=1
NEXTTIME=NTT+30
NTT=NEXTTIME
PROCSTEP=13
ENDIF
# phase 4.3: stand-by
IF PROCSTEP=13 AND TIME>=NEXTTIME
phase=4.3
pmpefl=0
air=0
n2=0
was=0
NEXTTIME=NTT+1
NTT=NEXTTIME
PROCSTEP=0
ENDIF
```

Annex 3: AS cycle modifications

Day 2: Addition of anoxic phase

Denitrification was taking place during settling, forming nitrogen gas bubbles and making the sludge to float. With the addition of an anoxic phase of 44 minutes after aeration, denitrification was expected to happen in the anoxic phase instead of in settling, in this way floating sludge would be avoided. In order to be able to add the anoxic phase maintaining a total cycle of 6 hours, the aerobic phase was reduced from 130 to 110 minutes, the settling was reduced from 60 to 40 minutes, and the sampling phase was eliminated.

Day 3: Addition of anoxic feeding and refresh stage

In general, in AS SBR reactors, COD is all up-taken during the anaerobic and aerobic phases, therefore the anoxic phase had to be fed with COD to ensure a carbon source for denitrification. As initially the COD pump was connected to the nutrients pump, in this first attempt at adding COD in the anoxic phase, the anoxic feeding was also including nutrients. To materialize this change, the 5 minutes anaerobic feeding was shortened to 3 minutes, and the other 2 minutes of feeding were added at the beginning of the anoxic phase. Also, a short aerobic phase of 10 minutes was added between the anoxic phase and the settling to refresh the biomass, avoiding anaerobic conditions during settling, which could lead to phosphate release. In this way, to maintain the length of the cycle in 6 hours, the anaerobic phase was reduced from 135 to 133 minutes, the aerobic phase was reduced from 110 to 100 minutes, and the anoxic phase was increased from 44 to 46 minutes. Furthermore, settling was decreased from 40 to 39 minutes because mistakenly, the cycle was lasting 361 minutes instead of 360 minutes.

Day 18: WAS corrected from 100 mL/cycle to 31 mL/cycle

It was realized that by mistake, WAS pump was working at a flow 3 times higher than the desired WAS (31 mL/cycle), it was discharging 100 mL/cycle. This meant that the SRT was 6 days instead of 20 days. The system had been running for 10 days like this, therefore all the biomass was already new and probably there were no nitrifiers because the SRT was too short. The flow of the WAS pump was corrected to 31 mL.

Day 28: Separation of COD pump, DO control introduced at 22% with gas valve

COD pump was separated from nutrients pump in order to be able to feed only COD during the anoxic phase.

In general, for DO control, this kind of bio-controllers inject nitrogen when DO is above the set point, but UNESCO-IHE nitrogen gas supply is limited. Therefore, some experiments were performed to be able to control DO without nitrogen injection. As the bio-controller in use had no more channels available for connection of additional devices, another bio-controller was needed. This implied the operation of two bio-controllers at the same time, and therefore, two cycles running simultaneously. A gas valve supplied with nitrogen was connected to a channel of the new bio-controller; signals of on and off were given through

statements in the computer. DO control at 22% (2 mg/L) was introduced in the aeration phase through statements for the original bio-controller. The option of delayed start of the cycles was used to be able to start both cycles exactly at the same time.

Day 30: pH settings adjustment and gas valve removal

High amounts of acid and base were being injected in the reactor in short periods. Therefore, the pH settings of the bio-controller were adjusted. P-value was reduced from 50 to 25 and the dead zone was increased from 0.05 to 0.10. These two measures made the system to inject less acid and base when the pH was out of range, and extended the pH variation range.

Regarding the nitrogen injection with the new bio-controller, it was realized that it was simpler to use the nitrogen supply from the new bio-controller instead of using a channel with the gas valve, therefore the statements were modified to achieve this.

Day 35: Aeration duration increased from 100 to 150 min, anaerobic duration decreased from 133 to 83 min, and addition of fresh biomass

In an attempt to enhance nitrification, the duration of the aeration phase was increased from 100 to 150 minutes, and in order to maintain the cycle in 6 hours, the anaerobic was decreased from 133 to 83 minutes. Also, 20 mL of seed sludge from Garmerwolde that was in the fridge were added to the reactor in order to add nitrifiers.

Day 36: SRT increased from 20 to 30 days and WAS reduced from 31 to 20 mL/cycle

Also in an effort to enhance nitrification, the total SRT was increased from 20 to 30 days. When the aeration was lasting 100 minutes and the total SRT was 20 days, the aerobic SRT was lasting 6 days. Now, with 150 minutes of aeration and 30 days of total SRT, the aerobic SRT was 13 days. This is certainly sufficient for nitrification, denitrification, and biological phosphorus removal at 20°C (Lopez Vazquez, 2016b). The increase of SRT was materialized with a reduction of the WAS flow from 31 to 20 mL/cycle.

Day 38: New start-up with fresh biomass

On day 38, the pH probe failed, it was reading zero, but once moved, a pH of 11 was measured. As the reactor overflowed due to the amount of base injected, some biomass was washed-out. Therefore, the basic biomass that remained in the reactor was taken out of it. A mixture of seed sludge from Garmerwolde which was in the fridge and backup sludge, were added to start-up the reactor again. The pH probe and the DO probe membrane were replaced.

Day 65: WAS reduced to 5 mL/cycle, actual SRT 30 days

Until day 65, the calculation of the WAS based on the SRT was not taking into account the solids which go out with the effluent and with the samples. Therefore, the WAS was overestimated, leading to an actual total SRT of 17 days instead of 30 days. To correct this, the WAS was reduced from 20 mL/cycle to 5 mL/cycle.

Day 74: DO increased from 22% to 50%

In an attempt to favour nitrification, DO was increased from 22% to 50%.

Day 86: Addition of alkalinity to the medium

According to Lopez Vazquez (2016a), 2 mol of alkalinity are consumed per mol of ammonia removed. Therefore, sodium carbonate was added to the nutrients medium to achieve this alkalinity concentration.

Day 87: WAS pump disconnected

During the first 3 months of operation, the WAS was automatically discharged with a pump in every cycle. It was realized that this modus operando was limiting the volume of samples possible to be taken, since to achieve a given SRT there is a maximum volume of sludge that can be weekly taken from the reactor. Therefore, it was decided to stop the WAS pump, and proceed with manual SRT control as in the AGS reactor.

Day 93: Extension of cycle duration and DO increased to 70%

Metcalf (2003) indicates the typical design parameters for an AS SBR with biological phosphorus removal, stating that the duration of anaerobic, aerobic, and anoxic phases are 1.5 to 3 hours, 2 to 4 hours, and 1 to 3 hours respectively. In a new attempt to favour nitrification, aeration duration was extended to the maximum recommended by Metcalf (2003). In general, the operation of reactors is easier when the length of the cycle is a multiple of 24 hours. Therefore, as with this extension the cycle would last 7.5 hours, some other phases were also extended to achieve a cycle of 8 hours. The anaerobic phase was extended from 83 to 90 minutes, aerobic phase from 150 to 240 minutes, anoxic phase from 46 to 69 minutes, refresh stage decreased from 10 to 9 minutes, and settling increased from 39 to 40 minutes. Furthermore, the DO was increased from 50% to 70%. The new HRT for this configuration was 16 hours.

Day 99: pH set point decreased from 7 to 6.85, and dead zone increased from 0.1 to 0.3

During the first months of operation, the set point for pH was 7 and the dead zone was 0.10. In an attempt to achieve nitrification, and to replicate the pH range of the AGS reactor, the pH set point was changed to 6.85, and the dead zone to 0.30 (with this change, the pH could vary from 6.60 to 7.10 since it seems there is a safety zone of 0.05).

Day 102: Mediums recipe corrected, anoxic phase shortened to 60 min

During the first month of operation, the AS mediums were slightly more concentrated than the AGS mediums since the feeding criteria used was to achieve the same concentration of COD and nutrients inside AGS and AS reactors. However, later on, the criteria was changed, deciding to have the same concentration of COD and nutrients in the influent of the reactors (including dilution), considering this a better criteria for the comparison of both reactors. As both reactors have the same dilution factors for their mediums, the recipe for the mediums would be the same. Nevertheless, a confusion with the printed recipes led to add the wrong amounts of chemicals to the mediums of the AS during 3.5 months of operation. This might have not affected the performance of the reactor, since the difference of concentration in the influent was not significant. The mistaken influent had 406 mg/L COD, 67 mg NH_4-N/L ammonium, and 10 mg PO_4-P/L phosphate, while the correct one had 366 mg/L, 60 mg NH_4- N/L, and 9 mg PO_4-P/L respectively.

Since the extension of the cycle length to 8 hours, the measured phosphate in the effluent was higher than at the end of the aerobic phase. Thus, some phosphate was being released in the anoxic phase, as it will be discussed in Chapter 4. In order to avoid phosphate release, the anoxic phase was reduced from 69 to 60 minutes, and the anaerobic from 90 to 99 minutes.

Day 108: Anoxic phase shortened to 46 min

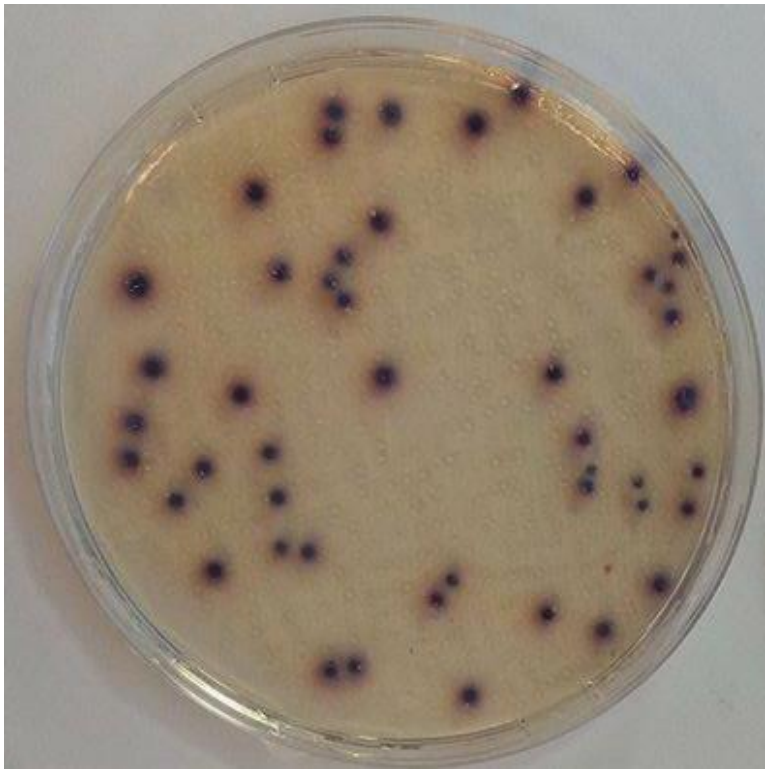
As phosphate in the effluent kept higher than at end aerobic, the anoxic phase was further reduced to its original duration, from 60 to 46 minutes, and anaerobic from 99 to 113 minutes.

Day 109: Dilution water changed to tap water

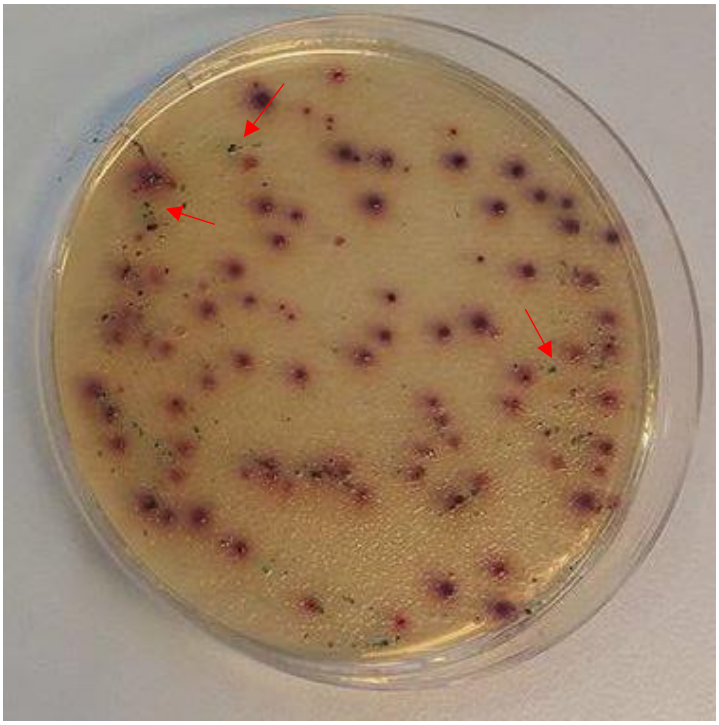
In a last attempt to achieve nitrification, as the AGS reactor was properly nitrifying when influent was diluted with tap water, AS reactor dilution water was changed to tap water. As the tap water has alkalinity and the mediums also had, plenty of acid was injected in the reactor in a short period of time. At some point, the reactor was overflowed and the acid bottle was emptied, so the pH increased to 10. This was the end of the AS reactor operation.

Annex 4: Microfiltration method

The Chromocult coliform agar plates plated with undiluted pottered sludge samples, presented in general, small particles which remained very small and turned into green after incubation. To determine whether these particles were attached to *E. coli* cells or not, a sample was filtered through a thick paper filter. The remaining particles in the filter were spread-plated, showing no growth of bacteria. These particles interfered with the counting of *E. coli* since in some cases they were over *E. coli* colonies, making unclear the number of colonies below them. Also, it could be observed that the *E. coli* colonies growing next to these particles acquired a lighter colour than usual, tending to pink. The closer the colonies were to the particles, the lighter the colour, which resembled the colour of other Coliforms. Therefore, it was difficult to distinguish *E. coli* colonies from other Coliforms. In these cases, in general, other Coliforms colonies were smaller in size than *E. coli* colonies, so the distinction was made based on this, thus, this counting was not as accurate as the others. An example is depicted in the following figure.



Normal blue-violet *E. coli* growth

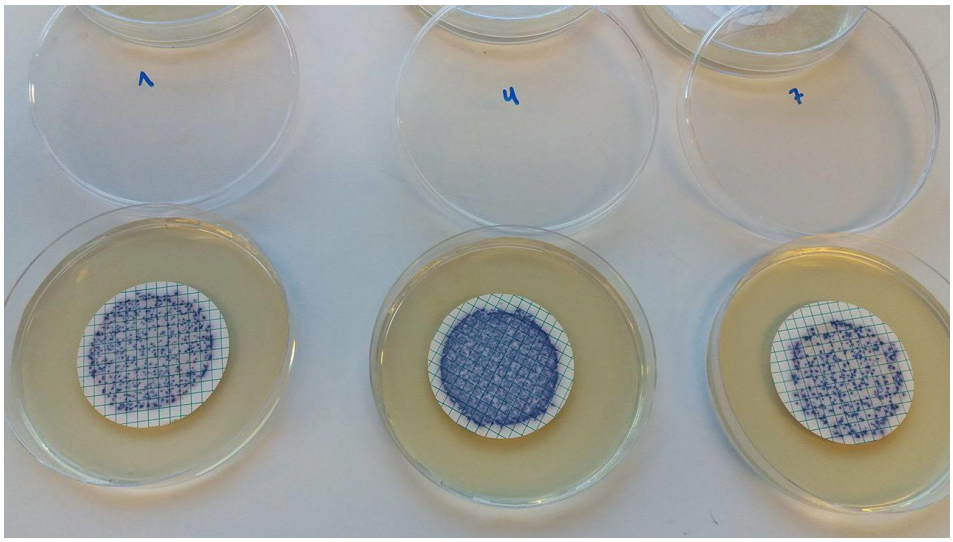


Small green particles (indicated with red arrow) interfering with counting and affecting colour of *E. coli*

Annex 4 - Figure 1: Particles in pottered sludge samples spread in Chromocult coliform agar

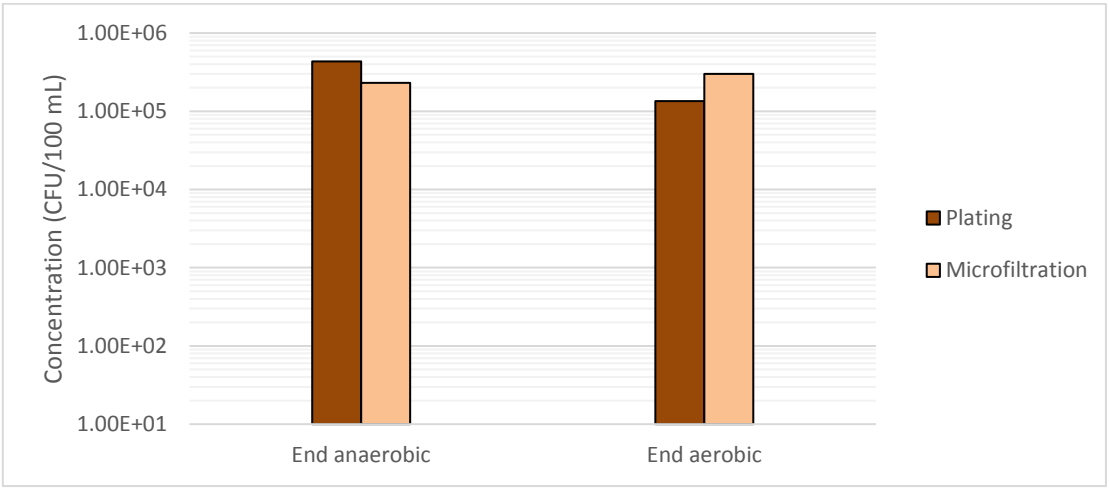
For samples with a low concentration of *E. coli*, the presence of particles was a problem, because the undiluted sample counting was not accurate because of the particles and the dilutions did not have enough colonies to consider the result reliable. Therefore, in order to get rid of these particles, the method "Microfiltration" was developed as described in the following paragraph.

First, a mixture of the sample with demi-water was filtered through three different filters: Whatman 0.45 µm, Whatman with thicker pores, and Filter 595 S&S; the filtered sample was collected and filtered again through the filters which come together with Endo NPS pack (these filters retain bacteria, they will be referred as Endo filters henceforth). Finally, the Endo filter was placed in Chromocult coliform agar plates. The resulting colonies in the Endo filters can be observed in the following figure, where 1 and 7 are the experiments for the first two filters previously mentioned (Whatman 0.45 µm and Whatman with thicker pores), and 4 corresponds to the last filter (Filter 595 S&S). Comparing the resulting number of colonies in the Endo filters, it could be observed that the experiments with the first two filters showed less *E. coli* than the third one, therefore *E. coli* was being retained by the first two filters. The result of the experiment with the third filter showed an uncountable number of CFU, so the correlation between this method and the conventional spread-plate technique could not be determined.



Annex 4 - Figure 2: "Microfiltration" experiment results

Thus, the experiment was repeated in another opportunity, but this time with 10-fold and 100-fold dilutions of two different samples in duplicates (end anaerobic - AEB and end aerobic – AEE). The 10-fold dilution samples were divided into three different Endo filters in order to obtain countable small amounts of CFU growing in each filter, and the 100-fold samples were divided into two different Endo filters for the same reason. Taking into account that the separation of supernatant and sludge is more accurate with settling plus filtering than only with settling, different results were expected between this method and the conventional spread-plate technique. With the spread-plate technique, the AEB sample showed more *E. coli* in the supernatant than in the sludge, and the AEE sample showed more *E. coli* in the sludge than in the supernatant. This means that if all the supernatant is removed with filtering, the AEB filtered sample will present less concentration of *E. coli* than with the spread-plate technique, and vice versa for the AEE sample. Indeed, this was the case. The results are shown in the following graph.



Annex 4 - Graph 1: Microfiltration experiments results

Regarding the samples taken at end anaerobic, the concentration for Microfiltration is 50% of the concentration for the spread-plate technique. In the case of end aerobic, the concentration for Microfiltration is 222% of the concentration for the spread-plate technique. It can be concluded that this method is reliable, but it should be taken into account that it gives a different result than the conventional spread-plate technique. After finishing these experiments, the amount of colonies obtained from the diluted samples with the spread-plate technique was enough to consider the results as reliable, thus, the application of this method was not further needed. If this method is going to be used in further studies, this experiment should be repeated to find a correlation between both methods.

Annex 5: TU Delft protocol for FISH analysis

Fluorescence In Situ Hybridization, FISH (lab protocol)

Steps involved:

Fixation

1. Harvesting cells
2. Washing cells
3. Fixing cells

Hybridisation

4. Coating slides
5. Immobilizing cells on the slide
6. Dehydrating of cells
7. Hybridization of cells
8. Washing of hybridized cells
9. Applying mounting medium and microscopic observation

Fixation (preparation of solutions see below)

Note 1: *Flocs, granules, aggregates, etc should be dispersed by syringing, pottering or short sonification.*
Note 2: *Not all type of cells will be pellet by centrifugation. Whether this is the case should be checked by filtration of the sample through a 0.2 µm membrane filter*
Note 3: *In cases of cells, which are grown extreme conditions (high pH, high salinity) it might be necessary to adjust the fixation buffer (PBS) correspondingly*

Fixing gram-negative cells:

1. Harvesting cells
Harvest cells by centrifugation or filtration

2. Washing
Wash and resuspend cells in 1x PBS or another appropriate wash buffer (note 3). (Washing means: centrifugation, removing supernatant and resuspending in washing buffer) Be sure to disrupt the pellet after centrifugation thoroughly in order to avoid large aggregates during FISH analysis.

3. Fixing cells
- Add three volumes of fixative (=fixation solution; see below for preparation) to one volume of suspension, keep on ice for 1-3 h (for example: if one volume of cell suspension is 0.5 ml then you should add 3 x 0.5 ml= 1.5 ml of fixative)
- Wash and resuspend cells in 1x PBS

- Add 1.2 volume 98 % ethanol (-20°C) to one volume of cell suspension (for example: 0.6 ml of 98% ethanol to 0.5 ml of cell suspension). The suspension should have an appropriate cell density, if not dilute or concentrate.
- Store samples at -20°C Make sure cells will not be frozen! (not solid, so that cell walls remain intact)

Fixing gram-positive cells:

- add 1.2 volumes of 98% ethanol to 1 volume of cell suspension. (for example: 0.6 ml of 98% ethanol to 0.5 ml of cell suspension)

Hybridization (preparation of solutions see below)

4. coating slides

Note 1: We use teflon coated microscope slides. The hydrophobic coating separates 6 wells preventing mixing of probes and hybridization buffer in nearby wells.

- Put gelatin coating solution in 70 °C water bath.
- Coat clean slides with gelatin by spreading 10 µl of heated (70°C) 0.1 % gelatin / 0.01% chromium potassium sulfate dodecahydrate solution in each well (use eppendorf-tip)
- Take of the coating (use eppendorf-tip) and dry the slides on warm lid of water bath.

Alternatively, the slides can be coated with a Poly L-Lysin coating:

- *Allow a diluted poly L-Lysin solution (0.01%) to come to room temperature (100 ml in coplin jars)*
- *Place slides in the coplin jars for 5 min*
- *Drain slides, dry 1 h at 60°C or overnight at room temperature in vertical position*

5. Immobilization of cell on microscope slides:

- Spread 2-15 µl cell suspension (depending on density) in each well of a gelatin coated teflon/glass microscope slide.
- Dry at 46°C until slides are dry

6. Dehydrating of cells

- Put dried slide(s) in a glass rack and fill three glass containers with respectively 50, 80 and 98% ethanol.
- Dehydrate cells by successive passage of the glass rack with slide(s) through 50, 80 and 98% ethanol (3 min each)
- Dry slides at room temperature (4 min) or if necessary at under air

If FISH is performed on gram positive (ethanol fixed) cells, the following two steps are required

- Put **10 µl** of **mutanolysin** (FLUKA 5000 U/ml in **0.1 M K_3PO_4** at pH 6.8) on each well at room temperature. Incubate about 30 minutes for newly fixed samples, for old cells 15 minutes is sufficient. **Use only ethanol fixed cells!**
- Wash afterwards with distilled water and repeat the dehydrating steps by successive passage through 50, 80 and 98% ethanol (3 min each)

7. Hybridisation of cells

- Prepare hybridization buffer with prescribed formamide concentration (depending on the used probes; see below) and keep at room temp.
- Thaw (= defreeze) oligonucleotide probes (working solutions)
- Prepare a hybridization tube by folding a unbleached tissue paper and put it into a 50 ml Falcon tube

- Pipette 10 μl hybridization buffer in each well with dehydrated cells
- Add 1 μl of (each) probe working solution (final concentration 5 pmol/μl for CY3 and CY5-labeled probes and 8.3 pmol/μl for FLUOS labeled probes) and mix with the hybridization buffer without scratching the teflon and cell layer. (If all samples on 1 slide are going to be hybridized with the same probecombination, it's easier to make a pre-mix of hybridization buffer + probes for all wells e.g. (10 μl + x·1 μl probe, x= number of wells)
- Pour the remaining hybridization buffer on the tissue paper in the hybridization tube
- Immediately transfer the slide into the hybridization tube and incubate for at least 1.5 h at 46°C
- In meantime prepare the washing buffer (see below) and preheat this buffer in a 48°C water bath.

8. Washing of hybridized cells

The next two steps should be performed as rapid as possible, since temperature fluctuations can cause unbinding of probes, resulting in weaker fluorescence signals.

- Rinse the hybridization buffer with the washing buffer from the slide, avoiding mixing of probe from one well to another.
- Transfer slide in the rest of the wash buffer, incubate 10-20 min at 48°C
- Remove washing buffer by rinsing with ice-cold demi-water and dry slides with pressed air

9. Applying mounting medium and microscopic observation

- Embed wells with Vectashield (= Amplifies fluorescence signal and avoids fading) by putting a tiny drop between 1&2, 3&4 and 5&6. Put a large cover slip (24mm x 60 mm) on slide
- Fix the cover slip with some nailpolish
- After drying of the nailpolish the specimens can be analyzed with the epi-fluorescence microscope

Preparation of solutions

Fixation of gram negative cells:

- **PBS (3x); 390 mM NaCl in 30 mM phosphate buffer (pH 7.2):** dissolve 0.49 g KH_2PO_4 in 80 ml, add 2.3 g NaCl and adjust pH to 7.2. Adjust the volume to 100 ml.
- **PBS (1x); 130 mM NaCl in 10 mM Phosphate buffer (pH 7.2):** Take 33 ml of PBS (3x) and adjust the volume to 100 ml with distilled water.
- **4% Paraformaldehyde in PBS** (see below for preparation, paraformaldehyde is very toxic, use gloves !!)
- **98% Ethanol** at -20°C
- **50%, 80% and 98% ethanol**
- **MilliQ** at 4°C
- **1 M NaOH:** Dissolve 4 g of NaOH in 80 ml distilled water, adjust the volume to 100 ml
- **1 M HCl**
- **Freshly prepared fixative (see below)**

Preparation of the fixative for gram negative samples:

Caution 1: paraformaldehyde is very toxic, WEAR GLOVES!!!!!
Caution 2: Use only freshly prepared fixative (< 24 h old), or deep frozen out of the -20°C.

- Put 0.4 g of paraformaldehyde in a 10 ml greiner vial
- Add 6.5 ml of milliQ and one drop of 1M NaOH. Close the vial with lid and heat to 60°C (Normally it is sufficient to warm it by hot running tap water)
- Shake vigorously until the solution has clarified (1-2 min.)
- Remove the solution from the heat source and add 3.3 ml of 3x PBS
- Adjust the pH to 7.2 with HCl (usually one drop 1 M HCl is sufficient)
- Filter the solution through 0.2 μm membrane disc filter
- Keep the solution on ice until used or store at -20°C

Hybridization:

- **0.1% gelatin solution in 0.01% chromium potassium sulfate dodecahydrate:** Dissolve 0.1 g gelatin and 0.01 g chromium potassium sulfate dodecahydrate in 100 ml milliQ.
- **5 M NaCl:** Dissolve 29.2 g NaCl in 80 ml milliQ and adjust the volume to 100 ml
- **1 M Tris/HCl (pH 8.0):** Dissolve 12.1 g Tris base in and adjust the pH to 8.0 with HCl, adjust the volume to 100 ml.
- **Formamide** (use formamide only in the fume and wear gloves!!!!)
- **0.5 M Na_2EDTA (pH 8.0):** Dissolve 18.1 g Na_2EDTA in 80 ml, adjust to pH 8.0 and adjust volume to 100 ml
- **10% (v/v) SDS:** Dissolve 2 g of sodiumdodecylsulfate in 20 ml of milliQ

Preparation of hybridization buffer for in situ hybridization at 46°C:

- Pipet into a 2 ml eppendorf:
  - 5 M NaCl 360 µl
  - 1 M Tris/HCl (pH 8.0) 40 µl
  - Add formamide and milliQ according to the following table:

% (v/v) Formamide	Formamide (µl)	MilliQ (µl)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400
65	1300	300

  - 10% (w/v) SDS 4 µl

Preparation of washing buffer for in situ hybridization at 46°C (washing at 48°C, 10-20 min)

- Pipet in a 50 ml Falcon tube and mix:
  - Tris/HCl (pH 8.0) 1 ml
  - Add 5 M NaCl and 0.5 M EDTA (pH 8.0) according to the following table:

% (v/v) Formamide in hybridization buffer	[NaCl] (mM)	5 M NaCl (µl)	0.5 M EDTA (µl)
0	0.900	9000	
5	0.636	6300	
10	0.450	4500	
15	0.318	3180	
20	0.225	2250	500
25	0.159	1590	500
30	0.112	1120	500
35	0.080	800	500
40	0.056	560	500

45	0.040	400	500
50	0.028	280	500
55	0.020	200	500
60	0.008	80	500
70	0.000	0	350

- Fill Falcon tube up to 50 ml with milliQ
- Add 50 μl of 10% (w/v) SDS
- Preheat the washing buffer at 48°C prior to use

Probe handling:

- Probes arrive freeze-dried (lyophilized). To each probe sterile milliQ is added to a final probe concentration of 100 pmol/μl. Shake at maximum speed in the thermostated (23°C) eppendorf shaker to dissolve (10 min, Molecular lab.)
- Prepare the working solution, final concentration 5 pmol/μl for CY3/5 and 8.3 pmol/μl for FLUOS. Mix two probes gently if required.
- Store probe stocks and working solutions at -20°C

Before the hybridization, thaw and store probes on ice.

Annex 6: Preparation and fixation procedure for DGGE and FISH analysis

For samples **other than granules and flocs** which contain sludge:

- Potter the sample. For 15 mL sample use 50 mL pottering tube. Granules should not be lost. All granules should be pottered, otherwise it can affect microscopy.
- Grab with gloves one fixation solution (toxic!) stored at -20°C. Leave it at room temperature to cool down.
- Grab 3 Eppendorf tubes 2 mL (DGGE, Gram positive, Gram negative).
- Put 2 mL of sample in each tube.
- Centrifuge for 3 minutes.
- Remove supernatant, check that all the volumes of sludge are the same.
- Add 1 mL of PBS (1x) to each tube.
- Mix carefully with pipette avoiding overflows.
- Centrifuge for 3 minutes.
- Remove supernatant.
- Store DGGE sample at -80°C.
- Add again 1 mL PBS (x1), mix with pipette, centrifuge, and remove supernatant.
- Add again 1 mL of PBS (x1) and mix with pipette.
    - Only for gram negative cells:
        - Put glove in hand with which the fixation solution will be manipulated.
        - In fumehood, transfer 0.5 mL (or 0.25 mL) sample to 1.5 mL (or 0.75 mL) fixation solution.
        - Put fixation solution with sample in cold box with ice for 1 - 3 hs.
        - Except for granules and flocs, centrifuge.
        - Remove supernatant.
        - Add 1 mL PBS (x1).
        - Mix with pipette.
        - Centrifuge.
        - Remove supernatant.
        - Add 1 mL PBS (x1).
        - Mix with pipette.
- In new Eppendorf tube, add 0.6 mL ethanol and 0.5 mL sample of gram positive, and in another one 0.6 mL ethanol and 0.5 mL sample gram negative.
- Store in vertical position at -20°C.

If **granules or flocs** are going to be fixated, use only 1 mL of sample, do not potter and do not centrifuge, just wash and let settle and remove supernatant with pipette. Instead of 1 - 3 hs, leave in ice with fixation solution overnight for the fixative to penetrate better into the granule.

Annex 7: Results of analyses

Removal efficiencies, results of chemical analyses, VSS/TSS results, and *E. coli* analyses results are presented in the following tables. Furthermore, the standard deviation (SD) of the measurements is presented when corresponds.

Annex 7 - Table 1: Removal efficiencies of chemical parameters - AGS

Day	AGS Removal efficiencies (%)			
	COD	**NH_4**	**PO_4**	**TN**
14	91.37	-	75.67	-
19	89.29	66.67	38.89	-
27	92.54	67.67	83.44	-
34	90.03	96.89	94.96	-
47	91.35	96.02	99.71	-
61	82.19	95.83	99.81	-
68	90.30	72.83	99.40	69.41
75	89.46	99.79	99.67	75.06
82	88.38	98.67	97.47	91.41
89	92.82	99.97	99.50	72.15
97	92.75	66.67	99.70	61.41
103	93.81	97.70	99.94	83.92
112	93.85	44.28	99.71	39.57
120	93.31	43.09	99.78	36.84

Annex 7 - Table 2: Chemical parameters concentrations - AGS

Day	Influent	End anaerobic	Effluent	SD-Influent	Theoretical influent
COD (mg/L)					
14	-	86.20	31.60	0.00	366.0
19	-	-	39.20	0.00	366.0
27	-	27.80	27.30	0.00	366.0
34	397.00	33.60	39.60	0.00	366.0
47	377.00	39.30	32.60	0.00	366.0
61	-	68.10	65.20	0.00	366.0
68	-	45.80	35.50	0.00	366.0
75	390.00	51.20	41.10	0.00	366.0
82	370.00	48.50	43.00	0.00	366.0
89	399.47	32.90	28.70	31.11	366.0
97	380.86	29.20	27.60	14.36	366.0
103	389.32	25.80	24.10	11.96	366.0
112	388.73	38.10	23.90	2.37	366.0
120	362.00	35.90	24.20	0.00	366.0
NH_4 (mg NH_4-N/L)					
14	-	-	-	-	-
19	-	47.00	20.00	0.00	60.0

Day	Influent	End anaerobic	Effluent	SD-Influent	Theoretical influent
27	-	40.80	19.40	0.00	60.0
34	64.30	28.20	2.00	0.00	60.0
47	59.00	40.20	2.35	0.00	60.0
61	-	37.00	2.50	0.00	60.0
68	-	36.30	16.30	0.00	60.0
75	60.30	27.10	0.13	0.00	60.0
82	58.60	23.00	0.78	0.00	60.0
89	62.55	25.50	0.02	1.92	60.0
97	48.24	29.10	16.08	0.09	60.0
103	55.65	20.90	1.28	0.97	60.0
112	63.53	44.20	35.40	1.53	60.0
120	60.10	46.10	34.20	0.00	60.0
PO_4 (mg PO_4-P/L)					
14	-	21.70	2.19	0.00	9.0
19	-	23.20	5.50	0.00	9.0
27	-	47.50	1.49	0.00	9.0
34	9.93	20.00	0.50	0.00	9.0
47	9.51	55.32	0.03	0.00	9.0
61	-	59.40	0.02	0.00	9.0
68	-	67.10	0.05	0.00	9.0
75	9.41	53.70	0.03	0.00	9.0
82	9.47	54.40	0.24	0.00	9.0
89	10.00	48.80	0.05	0.03	9.0
97	9.61	53.20	0.03	0.03	9.0
103	9.68	31.50	0.01	0.03	9.0
112	9.41	65.90	0.03	0.00	9.0
120	9.43	70.30	0.02	0.00	9.0

Annex 7 - Table 3: Nitrogen concentrations in effluent – AGS

Day	Effluent (mg N/L)		
	NO_3	**NO_2**	**TN**
68	0.62	1.43	18.35
75	14.42	0.49	15.04
82	3.55	0.71	5.04
89	16.80	0.60	17.42
97	1.71	0.83	18.62
103	4.36	3.31	8.95
112	1.71	1.28	38.39
120	2.56	1.20	37.96

Annex 7 - Table 4: VSS/TSS results – AGS

Day	Reactor					Effluent				
	TSS	SD-TSS	VSS	SD-VSS	VSS/TSS	TSS	SD-TSS	VSS	SD-VSS	VSS/TSS
	(g/L)				(%)	(g/L)				(%)
14	-	-	-	-	-	0.301	0.010	0.203	0.013	68
19	3.476	0.150	2.240	0.512	64	0.127	0.009	0.110	0.000	87
27	5.606	0.290	4.673	0.268	83	0.040	0.003	0.039	0.004	98
34	6.054	0.250	4.829	0.204	80	0.060	0.000	0.042	0.000	70
47	7.564	0.090	6.646	0.110	88	0.072	0.040	0.049	0.025	68
61	8.026	0.770	6.324	0.512	79	0.100	0.028	0.070	0.042	70
68	8.443	0.100	6.685	0.048	79	0.066	0.012	0.033	0.005	51
75	8.995	0.750	7.191	0.134	80	0.152	0.046	0.112	0.048	73
82	10.094	0.400	7.856	0.231	78	0.091	0.004	0.065	0.005	71
89	9.941	0.240	7.437	0.232	75	0.047	0.007	0.027	0.009	59
97	7.467	0.340	5.516	0.884	74	0.053	0.011	0.029	0.000	55
103	7.805	0.220	6.151	0.179	79	0.037	0.012	0.020	0.006	54
112	6.540	0.180	5.031	0.067	77	0.031	0.010	-	-	-
120	6.450	0.000	4.772	0.000	74	0.043	0.000	0.034	0.000	79

Annex 7 - Table 5: E. coli concentrations in influent and effluent and E. coli removal results - AGS

Day	*E. coli* (CFU/100 mL)				*E. coli* Log removal
	Influent	SD-Influent	Effluent	SD-Effluent	
61	1.17E+06	5.66E+04	1.00E+04	0.00E+00	2.07
68	5.15E+06	6.36E+05	1.00E+03	0.00E+00	3.71
75	4.50E+04	2.12E+04	1.00E+02	1.00E+02	2.65
82	3.95E+05	4.95E+04	9.50E+03	4.95E+03	1.62
89	4.19E+05	3.11E+04	1.00E+03	0.00E+00	2.62
97	9.50E+04	3.54E+04	8.26E+05	8.13E+04	0.00
100	4.80E+04	9.90E+03	3.40E+04	4.24E+03	0.15
105	1.26E+06	8.49E+04	7.07E+05	1.28E+05	0.25
110	1.58E+06	2.83E+04	5.55E+05	7.07E+03	0.45
120	1.47E+06	1.42E+05	4.00E+03	1.41E+03	2.56

Annex 7 - Table 6: Fate of E. coli results – AGS

Day	*E. coli* (CFU/100 mL)							
	End anaerobic				End aerobic			
	Sludge	SD-Sludge	Supernatant	SD-Supernatant	Sludge	SD-Sludge	Supernatant	SD-Supernatant
61	1.00E+04	0.00E+00	1.00E+04	0.00E+00	-	-	-	-
68	2.20E+05	1.41E+04	3.75E+05	7.78E+04	-	-	-	-
75	5.00E+04	0.00E+00	5.15E+04	2.12E+03	3.00E+04	0.00E+00	1.50E+03	7.07E+02
82	8.00E+04	4.24E+04	1.67E+05	2.05E+04	1.00E+04	0.00E+00	1.20E+04	0.00E+00
89	5.55E+04	2.56E+04	1.65E+05	7.07E+03	6.50E+03	7.07E+02	2.00E+03	1.41E+03
97	4.91E+05	2.84E+05	4.48E+05	3.34E+04	6.70E+05	5.66E+04	3.93E+05	3.21E+04
100	4.40E+05	1.41E+04	3.25E+04	7.78E+03	3.40E+05	2.83E+04	3.55E+04	9.19E+03
105	1.40E+06	2.83E+05	1.07E+06	1.50E+05	9.30E+05	0.00E+00	7.60E+05	1.56E+05
110	9.35E+05	3.54E+04	1.15E+06	1.52E+05	6.20E+05	2.83E+04	5.48E+05	1.39E+04
120	4.35E+05	2.12E+04	5.55E+05	3.54E+04	1.35E+05	7.07E+03	7.50E+03	7.07E+02

Annex 7 - Table 7: Removal efficiencies of chemical parameters – AS

Day	AS Removal efficiencies (%)		
	COD	**NH_4**	**PO_4**
7	93.57	34.18	30.70
15	93.65	22.54	24.60
23	91.75	21.45	34.70
43	95.57	84.04	42.00
57	95.47	90.68	87.15
64	97.84	54.70	98.22
70	94.10	46.36	72.49
77	95.96	33.14	88.62
85	95.99	52.87	96.10
93	95.76	39.04	97.12
101	93.88	27.95	29.51
108	97.33	31.07	64.93

Annex 7 - Table 8: Chemical parameters concentrations – AS

Day	Influent	End anaerobic	End aerobic	Effluent	SD-Influent	SD-End anaerobic	SD-End aerobic	SD-Effluent	Theoretical influent
COD (mg/L)									
7	-	-	34.80	26.10	0.00	-	0.00	0.00	406.0
15	-	-	-	25.80	0.00	-	-	0.00	406.0
23	325.00	120.00	50.00	26.80	0.00	0.00	0.00	0.00	406.0
43	453.83	-	-	20.12	64.72	-	-	6.01	406.0
57	362.00	-	-	16.40	0.00	-	-	0.00	406.0
64	492.27	-	21.37	10.62	46.60	-	4.95	3.89	406.0
70	420.87	116.07	15.37	24.85	25.37	23.56	3.82	3.22	406.0
77	479.00	33.87	19.37	19.37	9.57	14.14	0.00	2.83	406.0
85	407.93	13.12	19.62	16.37	14.36	1.06	5.30	0.71	406.0
93	409.63	-	20.62	17.37	11.96	-	8.84	6.36	406.0
101	443.46	-	36.62	27.12	2.39	-	0.35	0.35	406.0
108	388.73	22.62	7.12	10.37	2.37	6.01	0.35	3.54	366.0
NH_4 (mg NH_4-N/L)									
7	-	-	37.30	44.10	0.00	-	0.00	0.00	67.0
15	-	-	-	51.90	0.00	-	-	0.00	67.0
23	55.00	47.00	42.30	43.20	0.00	0.00	0.00	0.00	67.0
43	57.00	-	-	9.10	0.00	-	-	0.00	67.0
57	25.96	-	-	2.42	0.00	-	-	0.00	67.0
64	-	-	33.36	30.35	0.00	-	0.58	0.72	67.0
70	-	40.50	32.86	35.94	0.00	0.59	2.10	1.33	67.0
77	66.97	57.38	49.92	44.77	0.68	2.42	5.55	0.83	67.0
85	69.76	46.41	34.44	32.88	1.03	3.16	0.21	0.12	67.0
93	52.52	-	37.34	32.02	0.06	-	0.23	0.06	67.0
101	80.19	-	52.50	57.77	0.97	-	0.60	3.85	67.0
108	53.34	39.13	31.99	36.77	0.49	0.75	0.03	0.23	60.0

Day	Influent	End anaerobic	End aerobic	Effluent	SD-Influent	SD-End anaerobic	SD-End aerobic	SD-Effluent	Theoretical influent
PO_4 (mg PO_4-P/L)									
7	-	-	4.81	6.93	0.00	-	0.00	0.00	10.0
15	-	-	-	7.54	0.00	-	-	0.00	10.0
23	8.76	12.50	2.90	5.72	0.00	0.00	-	0.00	10.0
43	8.82	-	-	5.12	0.01	-	-	0.05	10.0
57	6.85	-	-	0.88	0.00	-	-	0.00	10.0
64	7.33	-	0.13	0.13	0.03	-	0.05	0.05	10.0
70	9.67	34.33	1.78	2.66	0.15	0.40	0.46	0.03	10.0
77	10.61	36.57	1.28	1.21	0.08	0.80	0.00	0.00	10.0
85	10.93	34.26	0.51	0.43	0.13	0.55	0.33	0.05	10.0
93	8.60	-	0.43	0.25	0.05	-	0.00	0.05	10.0
101	11.62	-	1.14	8.19	0.00	-	0.05	0.28	10.0
108	9.43	49.03	0.51	3.31	0.02	0.73	0.02	0.05	9.0
NO_3 (mg NO_3-N/L)									
85	-	-	8.50	-	-	-	0.71	-	-
93	-	-	7.02	-	-	-	0.38	-	-
101	-	-	2.98	-	-	-	0.01	-	-
108	-	-	0.24	-	-	-	0.02	-	-

Annex 7 - Table 9: VSS/TSS results - AS

Day	Reactor					Effluent				
	TSS	SD- TSS	VSS	SD- VSS	VSS/TSS	TSS	SD- TSS	VSS	SD- VSS	VSS/TSS
	(g/L)				(%)	(g/L)				(%)
2	1.463	0.034	-	-	-	0.038	0.000	-	-	-
7	1.094	0.000	-	-	-	0.036	0.000	-	-	-
15	0.972	0.020	0.836	0.060	86	0.021	0.001	0.002	0.000	10
23	1.586	0.020	1.456	0.020	92	-	-	-	-	-
30	1.858	0.020	1.673	0.034	90	-	-	-	-	-
38	1.746	0.020	-	-	-	-	-	-	-	-
43	2.041	0.100	1.682	0.057	82	0.042	0.000	0.036	0.000	86
57	2.452	0.020	1.919	0.065	78	0.056	0.004	0.043	0.004	78
64	3.404	0.150	2.502	0.042	74	0.032	0.002	0.030	0.001	94
70	3.522	0.060	2.630	0.023	75	0.023	0.002	0.020	0.000	86
77	3.712	0.080	3.111	0.070	84	0.023	0.002	0.020	0.000	86
85	5.286	0.060	4.050	0.021	77	0.051	0.000	0.041	0.001	81
93	6.531	0.040	4.995	0.045	76	0.048	0.000	0.043	0.000	89
101	5.778	0.050	4.380	0.128	76	0.036	0.000	-	-	-
108	5.03	0.00	3.872	0.000	77	0.024	0.000	-	-	-

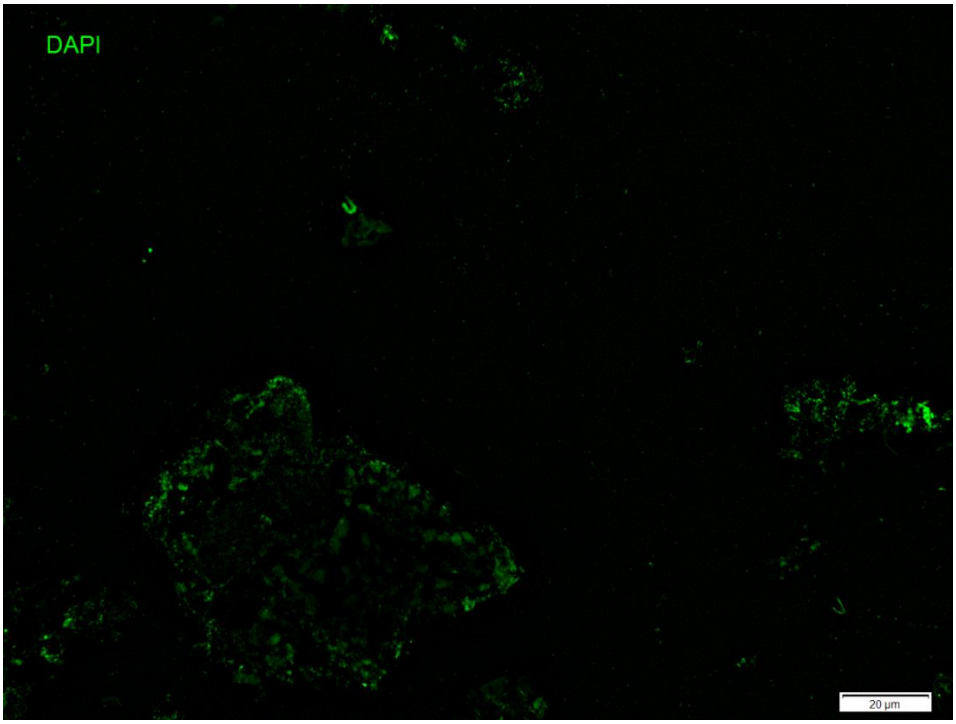
Annex 7 - Table 10: E. coli concentrations in influent and effluent and E. coli removal results - AS

Day	*E. coli* (CFU/100 mL)				*E. coli* Log removal
	Influent	SD-Influent	Effluent	SD-Effluent	
57	3.15E+07	2.12E+06	3.35E+07	2.12E+06	0.00
64	9.95E+06	1.48E+06	6.60E+06	7.07E+05	0.18
70	1.30E+06	1.84E+05	1.26E+06	2.12E+05	0.01
77	1.74E+05	7.07E+03	1.72E+05	1.48E+04	0.01
85	4.17E+06	1.04E+06	4.32E+06	5.59E+05	0.00
93	2.02E+06	7.07E+03	1.73E+06	4.43E+04	0.07
99	1.49E+06	1.68E+05	1.12E+06	2.62E+05	0.12
106	2.30E+05	2.83E+04	2.23E+05	2.09E+04	0.01

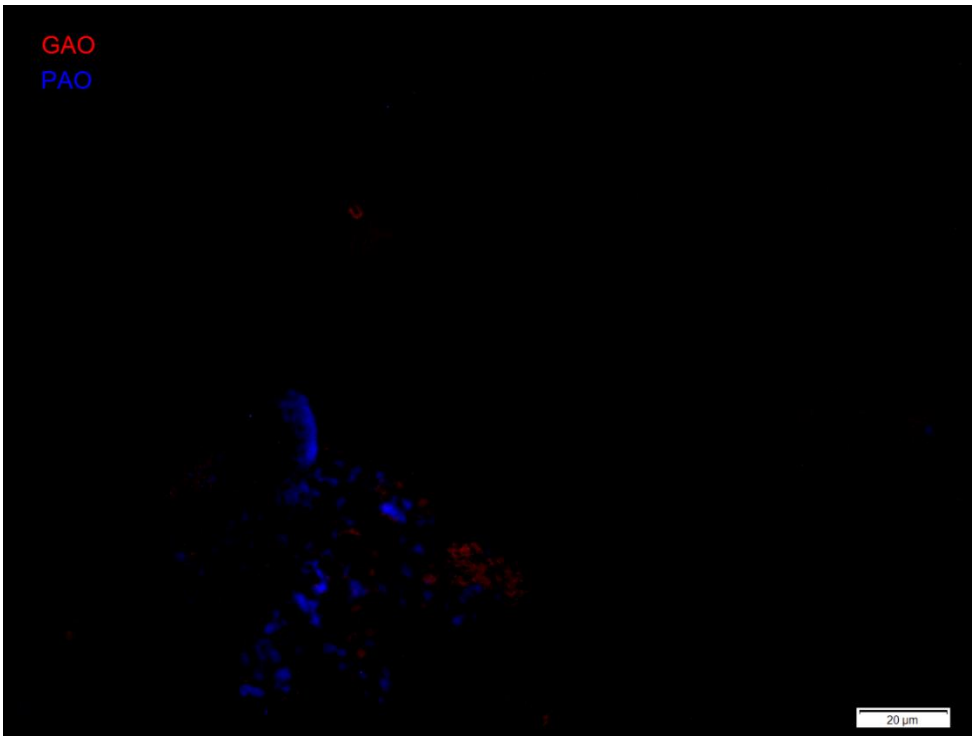
Annex 7 - Table 11: Fate of E. coli results - AS

Day	*E. coli* (CFU/100 mL)							
	End anaerobic				End aerobic			
	Sludge	SD-Sludge	Supernatant	SD-Supernatant	Sludge	SD-Sludge	Supernatant	SD-Supernatant
57	4.15E+07	3.54E+06	2.50E+07	1.41E+05	-	-	-	-
64	3.45E+06	7.07E+04	5.30E+06	1.41E+05	-	-	-	-
70	1.40E+06	9.19E+04	1.32E+06	3.54E+04	1.31E+06	1.98E+05	1.44E+06	6.36E+04
77	5.65E+05	9.19E+04	1.02E+05	0.00E+00	5.70E+05	9.90E+04	1.00E+05	4.24E+04
85	2.76E+06	3.54E+04	4.12E+06	5.59E+05	2.70E+06	2.40E+05	4.36E+06	4.53E+05
93	-	-	-	-	1.51E+06	9.90E+04	1.74E+06	9.90E+04
99	1.85E+06	3.40E+05	1.95E+06	0.00E+00	1.25E+06	7.48E+04	1.29E+06	2.43E+05
106	3.50E+05	1.41E+04	2.05E+05	3.55E+04	2.50E+05	1.41E+04	2.19E+05	3.06E+03

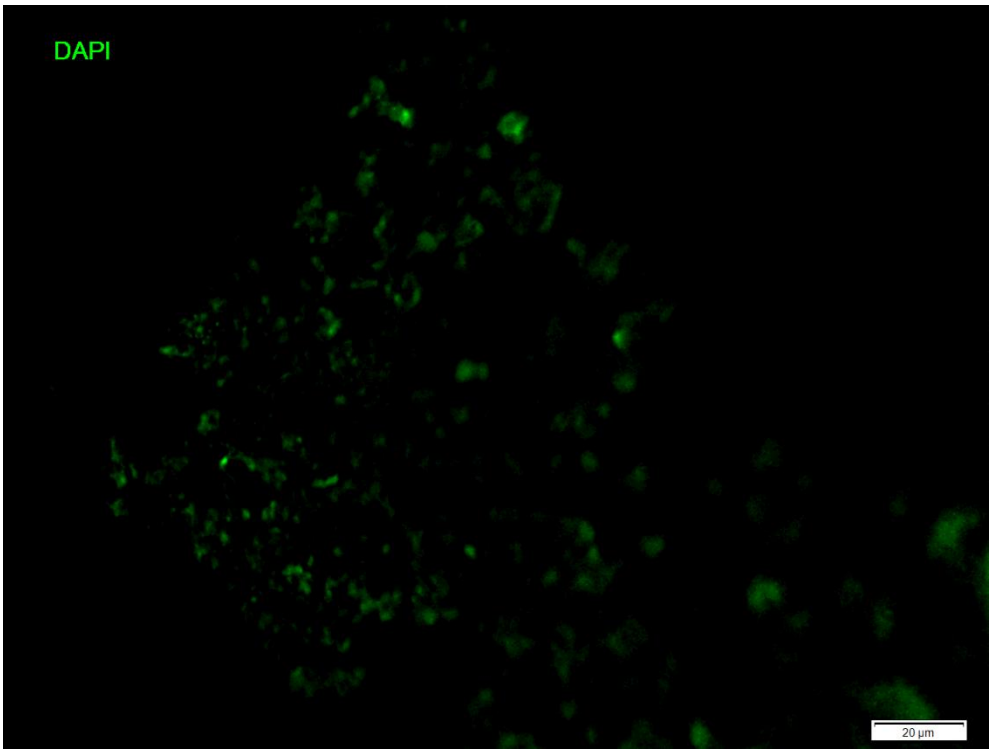
Annex 8: PAO’s and GAO’s FISH results



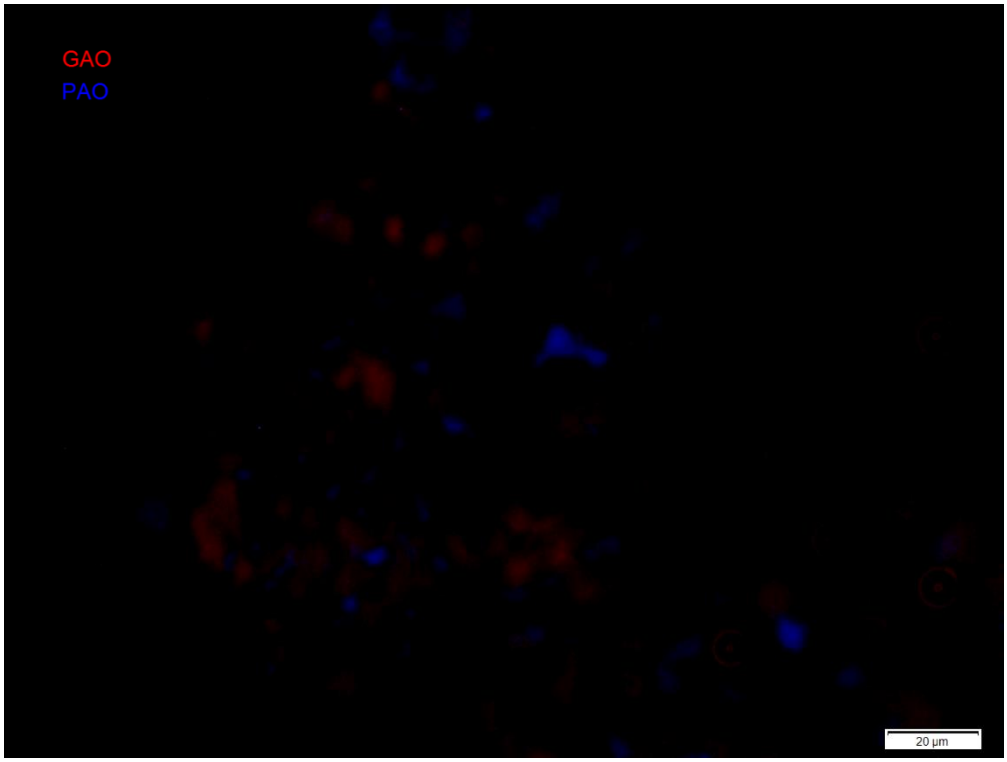
Annex 8 - Figure 1: PAO’s and GAO’s FISH results for AGS - Sample pottered AGS, end anaerobic – All DNA



Annex 8 - Figure 2: PAO’s and GAO’s FISH results for AGS - Sample pottered AGS, end anaerobic – Overlaying (Red: GAO’s, blue: PAO’s)



Annex 8 - Figure 3: PAO's and GAO's FISH results for AS - Sample pottered AS, end anaerobic – All DNA

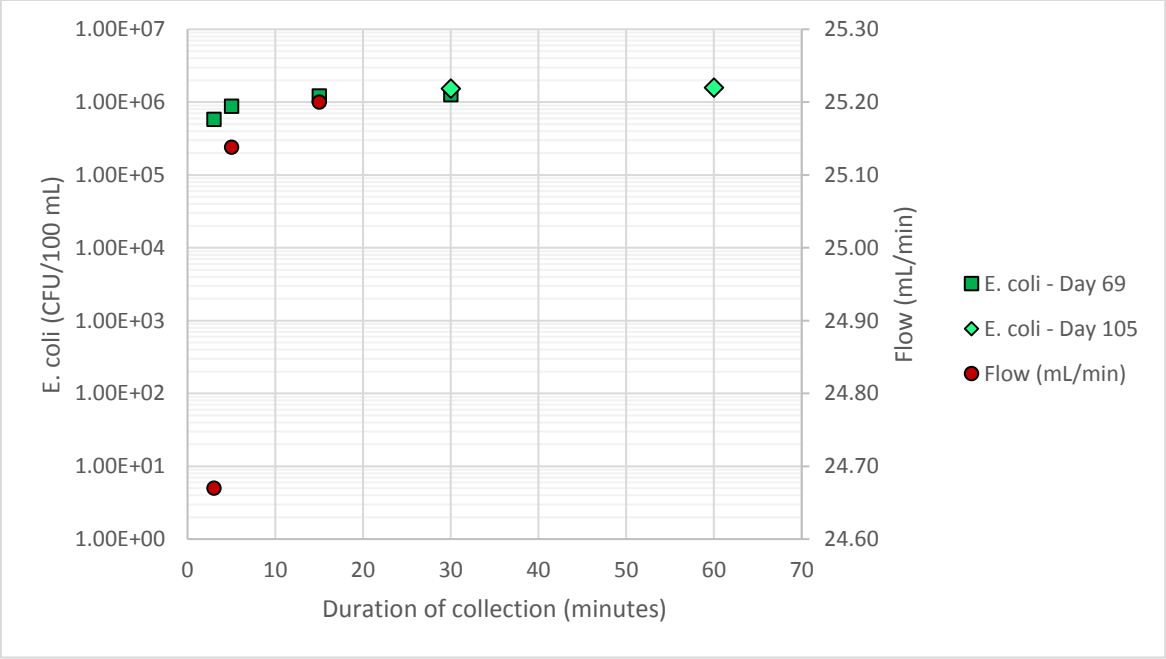


Annex 8 - Figure 4: PAO's and GAO's FISH results for AS - Sample pottered AS, end anaerobic – Overlaying (Red: GAO's, blue: PAO's)

Annex 9: Other *E. coli* experiments for AGS

Regarding the sampling procedure of the influent, at first this was collected only for approximately 1 minute in a beaker, and then a sample was taken from this. Afterwards, it was realized that the flow of the pumps per minute changed if the collection lasted more time, and therefore the concentration of the parameters would also be different. This was especially noticed in the AS reactor, but it led to change to a longer collection time of the influent also for the AGS reactor, thus, it was increased to 3 minutes.

As mentioned before, on days 97 and 100, *E. coli* influent concentration was unexpectedly lower that the rest of the sampling points. This led to review again the sampling procedure of the influent; an experiment was performed in order to find a correlation between the *E. coli* concentration and the duration of influent collection. Different times of collection were set, being the maximum equal to the total length of the feeding: 3, 5, 15, 30, and 60 minutes. Influent was collected 5 different times with this different collection durations, and samples were taken. Due to limitations in the available time for the experiment (120 minutes; while the reactor is not in feeding phase), the durations 3, 5, 15, and 30 were experimented in one opportunity, and the duration 60 together with the repetition of 30 was tested in another one. The results are shown in the following graph, where also the differences of flow per minute for durations 3, 5, and 15 are shown.

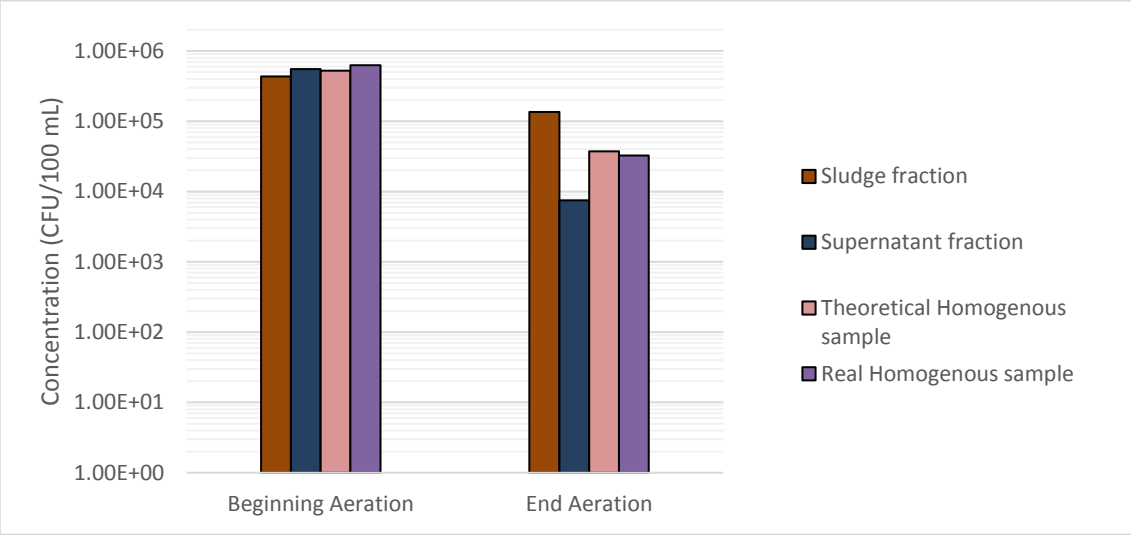


Annex 9 - Graph 1: E. coli concentration in influent as a function of influent collection duration - AGS

From the previous graph, it can be observed that *E. coli* concentrations for 30 minutes of duration for the different experimental days, are almost the same, thus, it will be assumed that all data corresponds to the same experimental day. It can be observed that the

concentrations for 15, 30, and 60 minutes are quite similar, therefore, from that day onwards, the collection of the influent was done in 15 minutes.

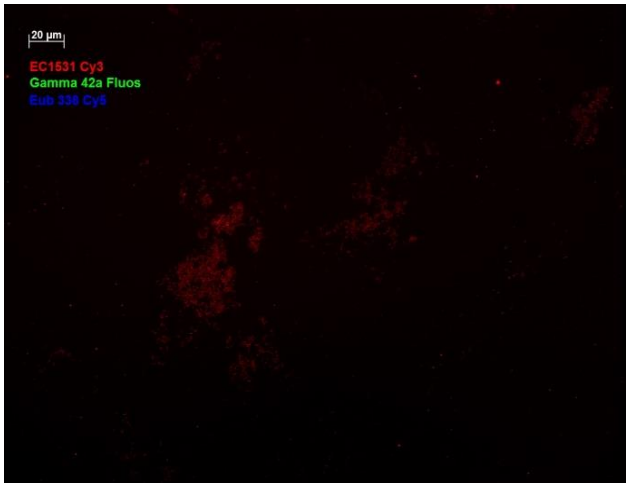
As mentioned in Chapter 3, in some specific weeks, apart from the routine samples, a homogenous sample of pottered granules and supernatant was taken at end anaerobic and aerobic; this was done to check the *E. coli* concentration of this sample with its respective fraction of pottered granules and supernatant separately. Taking into account the volume of sludge and the volume of supernatant in the homogenous sample, and the *E. coli* concentrations in the sludge and supernatant fractions from the separate samples, the theoretical *E. coli* concentration of the homogenous sample was calculated according to Section 3.1.5, and compared to the real concentration. The results are given in the following graph.



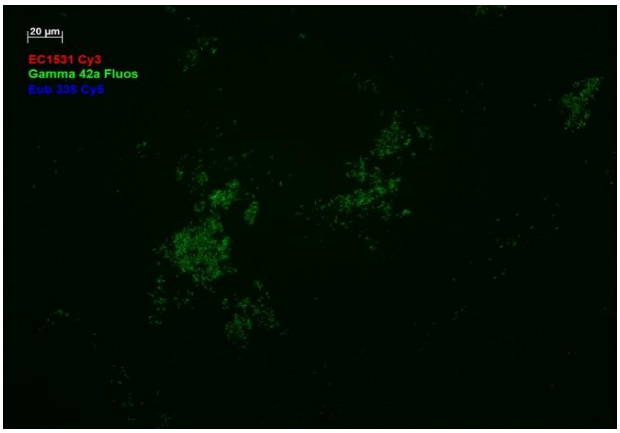
Annex 9 - Graph 2: Comparison of homogenous and separate samples

It can be stated that the theoretical and the real value are quite similar; the differences might be attributed to inaccurate measurement of volumes, loss of sludge stuck to the potter tube and to the sampling cups during the pottering procedure, and the difficulty of obtaining a homogenous sample when plating a pottered sample (there are still particles of different sizes).

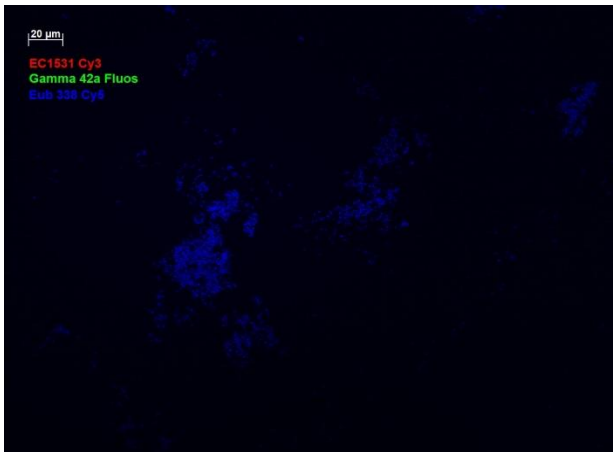
Annex 10: *E. coli* FISH results



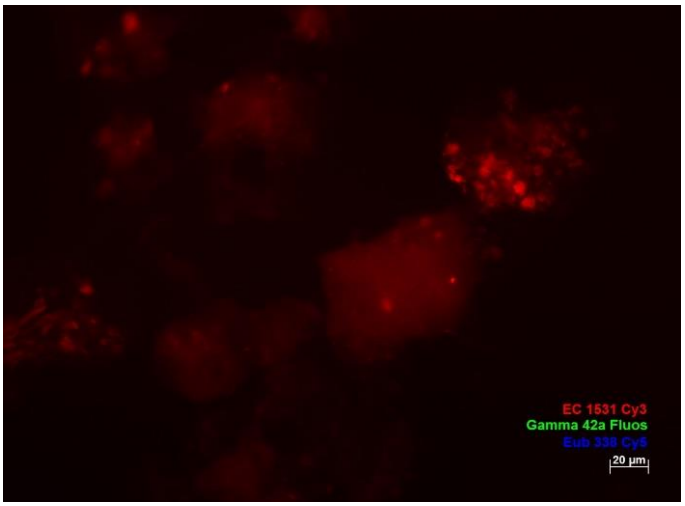
Annex 10 - Figure 1: E. coli FISH results for AGS – E. coli Pure culture – Probe EC 1531



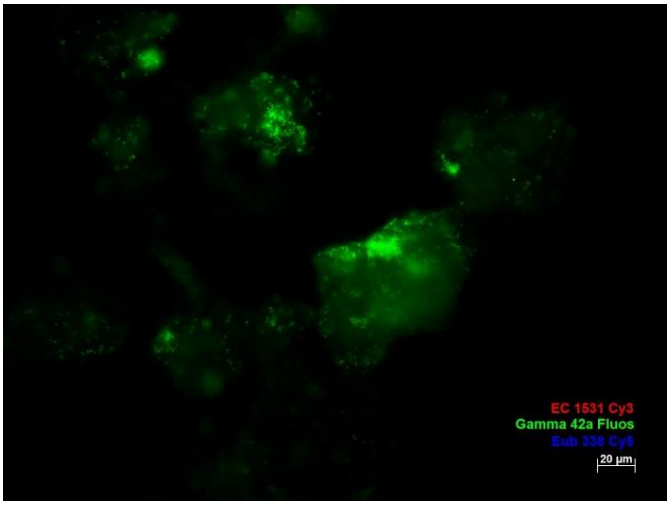
Annex 10 - Figure 2: E. coli FISH results for AGS – E. coli Pure culture – Gamma-proteobacteria



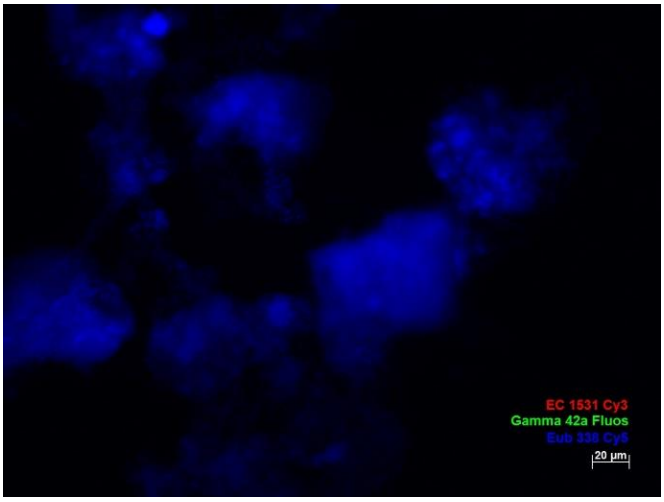
Annex 10 - Figure 3: E. coli FISH results for AGS – E. coli Pure culture – All bacteria



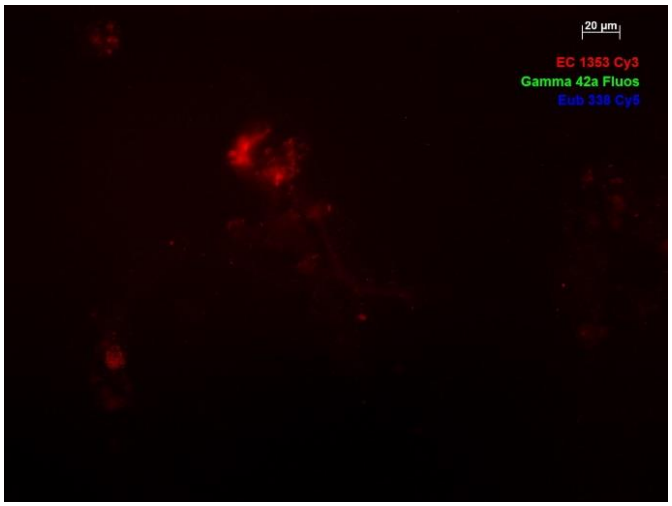
Annex 10 - Figure 4: E. coli FISH results for AGS - Negative control - Probe EC 1531



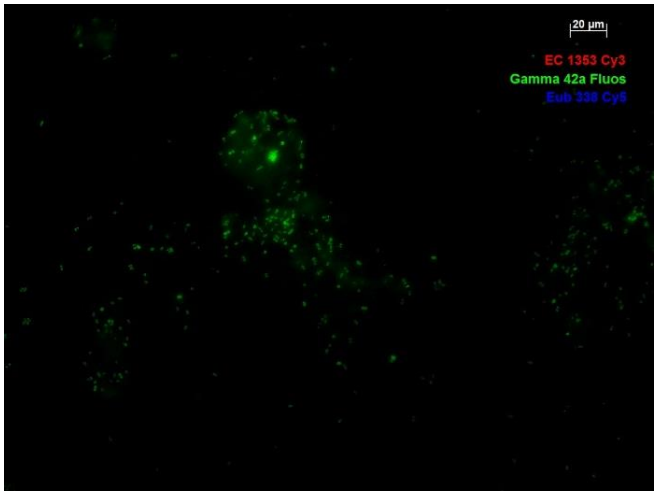
Annex 10 - Figure 5: E. coli FISH results for AGS - Negative control - Gamma-proteobacteria



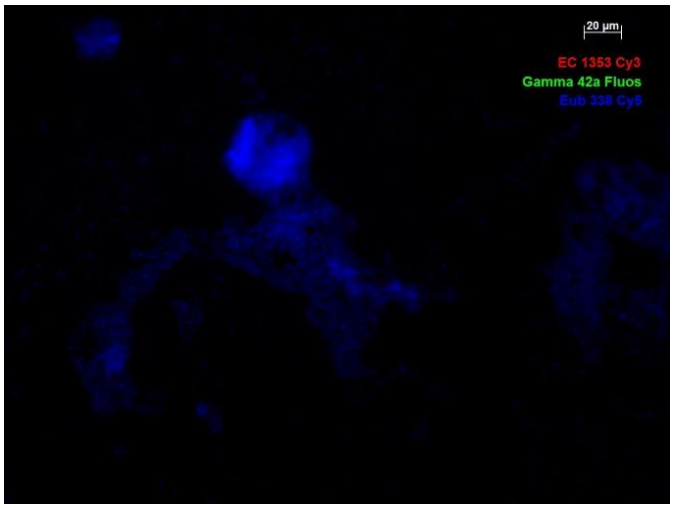
Annex 10 - Figure 6: E. coli FISH results for AGS - Negative control – All bacteria



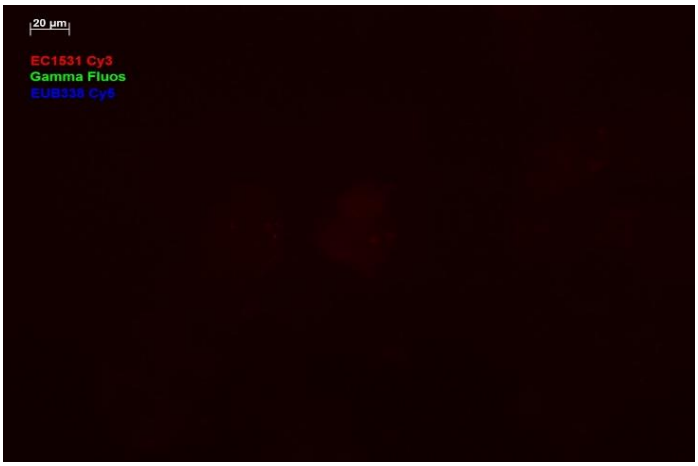
Annex 10 - Figure 7: E. coli FISH results for AGS - Negative control and pure culture - Probe EC 1531



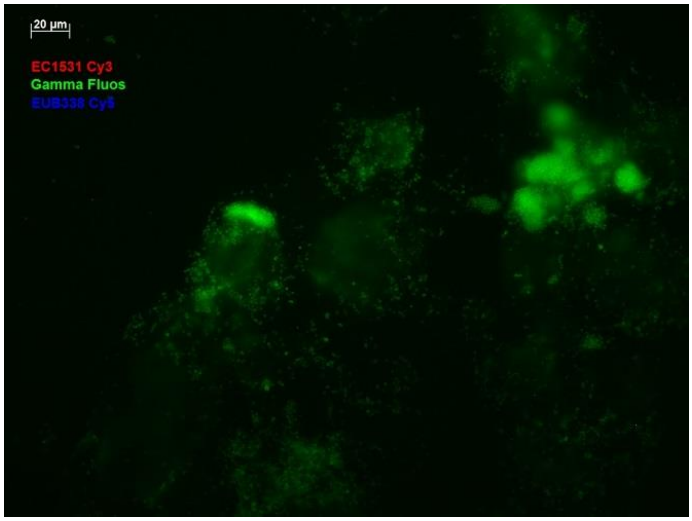
Annex 10 - Figure 8: E. coli FISH results for AGS - Negative control and pure culture - Gamma-proteobacteria



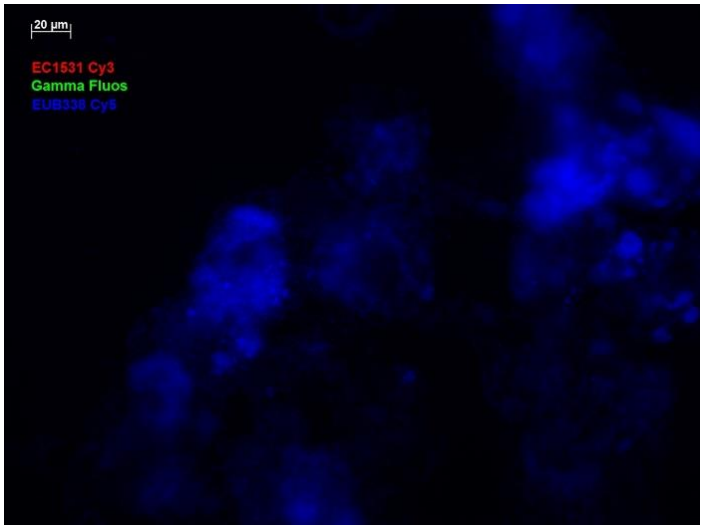
Annex 10 - Figure 9: E. coli FISH results for AGS - Negative control and pure culture - All bacteria



Annex 10 - Figure 10: E. coli FISH results for AGS - Sample pottered AGS, end anaerobic - Probe EC 1531



Annex 10 - Figure 11: E. coli FISH results for AGS - Sample pottered AGS, end anaerobic - Gamma-proteobacteria



Annex 10 - Figure 12: E. coli FISH results for AGS - Sample pottered AGS, end anaerobic – All bacteria