THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Removal of Organic Micropollutants from Wastewater in Biofilm Systems

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Cover:
Light microscopy image of aerobic granules dominated by fungi at 2 x magnification.
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ABSTRACT
The presence of organic hazardous substances in the aquatic environment, such as pharmaceutically active compounds and personal care products, has become a worldwide issue of increasing environmental concern. Present at concentration of nano- to milligram per liter, they are defined as organic micropollutants (OMPs). Wastewater treatment plants (WWTPs) have been recognized as the main route of emission of OMPs into the environment and as hotspot for antibiotic resistance. Not being designed for the elimination of micropollutants, the removal is often incomplete, resulting in continuous discharge. Therefore, research currently focuses on the enhancement of conventional WWTPs via physical-chemical and biological treatment processes. Among biological processes, biofilm-based treatment technologies have been found more efficient in the biotransformation of OMPs than conventional activated sludge treatment processes. Aerobic granular sludge (AGS) is a form of free-floating biofilm technique for simultaneous removal of organic carbon, nitrogen, and phosphorus in a single process step. The longer solid retention time, the higher concentration and microbial diversity and the presence of micro-niches of different redox conditions are features of AGS that make this system very attractive for the removal of OMPs. An in-depth understanding of the fate of OMPs in such systems under different operational conditions is still required. The present work investigates the degradation mechanisms of OMPs in biomass from both full-scale treatment plants and laboratory reactors. Specifically, it focuses on the impact of different conformations of AGS on the sorption of selected pharmaceuticals and the potential of different biofilm systems at the full scale WWTP to eliminate OMPs.

Keywords: Organic Micropollutants; Pharmaceuticals; Aerobic Granular Sludge; Sorption; Biological Treatment

PREFACE

This licentiate thesis is based on the research performed in the Division of Water Environment Technology (Chalmers University of Technology) between August 2017 and June 2019 under the supervision of Britt-Marie Wilén, Oskar Modin and Frank Persson. This research has been funded by the Swedish Research Council FORMAS.

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ii. **Burzio C.**, Nivert E., Mattsson A., Svahn O., Modin O., Persson F., Wilén B.M. Removal of organic micropollutants in the biological units of a Swedish wastewater treatment plant. *IWA World Water Congress, Copenhagen, Denmark.*

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LIST OF ACRONYMS AND ABBREVIATIONS

AGS: aerobic granular sludge

AOB: ammonia oxidizing bacteria

COD: chemical oxygen demand

DO: dissolved oxygen

EPS: extracellular polymeric substance

HRT: hydraulic retention time

MATS: microbial adhesion to solvents

MBBR: moving bed biofilm reactor

NOB: nitrite oxidizing bacteria

OLR: organic loading rate

OMP: organic micropollutant

PAO: polyphosphate accumulating organism

PHAs: polyhydroxyalkanoates

SBR: sequencing batch reactor

SRT: solid retention time

TN: total nitrogen

TOC: total organic carbon

VFA: volatile fatty acid

WWTP: wastewater treatment plant

Contents

1. Introduction

The liquid waste generated by a human community – wastewater – constitutes the water consumed by the urban and industrial activities, which has been contaminated by a variety of pollutants, such as nutrients, particles, toxic compounds, and pathogenic microorganisms. If released untreated, wastewater could cause the deterioration of the receiving aquatic environment and lead to epidemic outbreaks. Wastewater treatment objectives have evolved during the history (Metcalf & Eddy, 2004). Until the 1970s, the removal of colloidal and suspended material, pathogens and biodegradable organics was the primary concern. Removal of nitrogen and phosphorus, began to be addressed in the 1980s, with a continuation and improvement of the previous objectives. Studies performed in the 1990s revealed that sexual disruption in fish occurred as a consequence of exposure of estrogen present in the effluent of treated wastewater (Purdom et al., 1994; Routledge et al., 1998). Since the end of the last century, the presence of organic hazardous substances in the aquatic environment has become a worldwide issue of increasing environmental concern (Halling-Sørensen et al., 1998; Ternes, 1998). Pharmaceutically active compounds, personal care products, pesticides, synthetic and natural hormones, and industrial chemicals belong to this group of concerning emerging contaminants, which are typically detected in aquatic environments and wastewater at low concentrations (ng/L–µg/L). Therefore, they are referred to as organic micropollutants (OMPs) or trace organic contaminants.

Wastewater treatment plants (WWTPs) have been recognized as one of the main pathways of release of OMPs into the environment (Luo et al., 2014) and as hotspot for antimicrobial resistance (Berendonk et al., 2015). The regulation of the release of OMPs from WWTPs into the environment is a topic of discussion and the plants might face in the coming future new treatment objectives concerning the emissions of those substances (Rizzo et al., 2019).

1.1. Biological wastewater treatment processes

Municipal WWTPs typically include a preliminary and a primary treatment step, a secondary biological treatment process and a tertiary treatment level. While in the preliminary treatment, coarse materials and debris are separated via screening, in the primary treatment suspended solids and organic matter are removed in sedimentation tanks. In secondary treatment biodegradable organic matter, nutrients and pathogens are degraded in biological reactors. Biological treatment steps rely on natural microbiological processes, in which microorganisms convert conventional pollutants into less harmful metabolites such as carbon dioxide, water and nitrogen gas and into new microbial biomass. Separation methods, such as settling, or filtration are then used to separate biomass and to uncouple the hydraulic retention time from the solid retention time. Disinfection and removal of suspended solid is part of a tertiary step.

1.1.1. Main biological processes and microbiology

Biological treatments in WWTPs generally consist of the removal of organic matter, nitrogen and phosphorus through a combination of aerobic, anoxic and anaerobic tanks, which provide the adequate redox conditions necessary for the targeted microorganisms to grow and thrive. The function of a biological reactor relies on the collective metabolic activities of the constituent microbial community members (Wagner et al., 2002). The microbiome found in wastewater includes Bacteria, Archaea, Fungi, Protists, and Metazoa. Protists and metazoa are the main predators of bacteria and together with bacteriophages contribute to the regulation of

the bacterial population, which constitutes the dominant group within the biological community in wastewater treatment systems (Feng et al., 2017).

The degradation and mineralization of organic matter is performed by chemoorganoheterotrophic bacteria, which metabolize organic carbon as source of both electrons and carbon. Aerobic organisms activate hydrocarbons by initial hydroxylation via oxygenase-mediated metabolism, involving either monooxygenase or dioxygenase enzymes (Rieger et al., 2002).

The removal of nitrogen is performed under aerobic and anoxic conditions by autotrophic and heterotrophic bacteria in processes referred to as nitrification and denitrification respectively. During nitrification, ammonium (NH_4^+) is oxidized by nitrifying bacteria to nitrate (NO_3^-), through two sequential microbial oxidation processes. In nitritation, aerobic ammonia oxidizing bacteria (AOB) and archaea (AOA) oxidizes ammonium into hydroxylamine (NH_2OH) using the enzyme ammonia monooxygenase (AMO). This is followed by NH_2OH oxidation to NO_2^-, catalyzed by the enzyme hydroxylamine oxidoreductase (HAO). In nitratation, nitrite is oxidized to nitrate in the presence of oxygen by nitrite oxidizing bacteria (NOB) using the nitrite oxidoreductase (NXR) enzyme. Denitrification is the reduction of NO_3^- or NO_2^- (used as electron acceptor) to dinitrogen gas (N_2) under anoxic conditions by heterotrophic bacteria, which oxidizes biodegradable organic carbon for energy source (electron donor). This pathway requires four different enzymes, including nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos). Nitrogen removal can also be performed by anaerobic ammonium oxidation (anammox) bacteria, which anaerobically oxidize ammonium and reduce nitrite producing dinitrogen gas.

Biological removal of phosphorus is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up phosphate intracellularly in the form of polyphosphate (polyP) granules when exposed to alternating anaerobic and aerobic environments and/or anoxic conditions. In the anaerobic phase, organic matter is fermented into volatile fatty acids (VFAs). The VFAs are then stored intracellularly as polyhydroxyalkanoates (PHAs) by PAOs. The energy necessary for this biotransformation is provided by the breakdown of the two intracellularly stored polymers polyP and glycogen (gly), which enables phosphate (PO_4^{3-}) to be released. The stored PHAs are later used as an energy source by PAOs in the aerobic phase for PO_4^{3-} uptake and poly-P storage. Finally, PO_4^{3-} is removed from the wastewater by removing the poly-P rich biomass. When exposed to anoxic conditions, nitrogen and phosphorus can be simultaneously removed by denitrifying PAOs (d-PAOs), which use nitrate as final electron acceptor instead of oxygen. Nitrate existing in the anaerobic stage could be used as an electron acceptor for the growth of denitrifying non-polyP heterotrophs. Their presence reduces the amount of substrate available for PAOs, and hence causes the reduction of phosphorus release.

1.1.2. **Biomass configuration**

Biological wastewater treatment technologies can normally be categorized in (i) suspended growth systems and (ii) attached growth systems. In suspended growth systems (activated sludge), the microorganisms grow in flocs, which are retained in the systems by separation from the effluent through filtration or sedimentation and recirculation in the system. In the attached growth system (biofilm systems), microorganisms can grow on different support materials or can self-aggregate as biofilm and be retained by their own density. Trickling filter

and rotating biological contactors are examples of attached growth systems onto fixed film, while Moving Bed Biofilm Reactors (MBBRs) are attached growth systems with suspended biofilm in the reactor by means of mechanical mixing or aeration.

Aerobic Granular Sludge

Granular sludge is recognized as a special case of biofilm, where self-immobilized microorganisms embedded in a three-dimensional network of Extracellular Polymeric Substances (EPS) form dense, compact, fast settling aggregates. EPS are metabolic compounds, constituted mainly by polysaccharides, proteins, humic acid, uronic acids and phospholipids (Adav et al., 2008a). EPS are responsible for the mechanical stability of biofilms, interconnecting and immobilizing bacterial cells. This scaffold protects the microorganisms from external shocks, such as starvation, desiccation, toxic compounds (e.g., antibiotics, metals), ultraviolet radiation, predation from some protozoan grazers (Flemming and Wingender, 2010). The distribution and the proportion of the different fractions of polymers determine the physico-chemical characteristic of the granule (Seviour et al., 2012). This biofilm structure incorporates water channels that allows transport of nutrients and electron acceptors. Within the granule a redox and substrate gradient is created which is fundamental for the coexistence of a diverse population of microorganisms (Aqeel et al., 2019). Hence, nitrification, denitrification, biological phosphorus removal and organic matter mineralization can occur simultaneously within the granule (Figure 1).

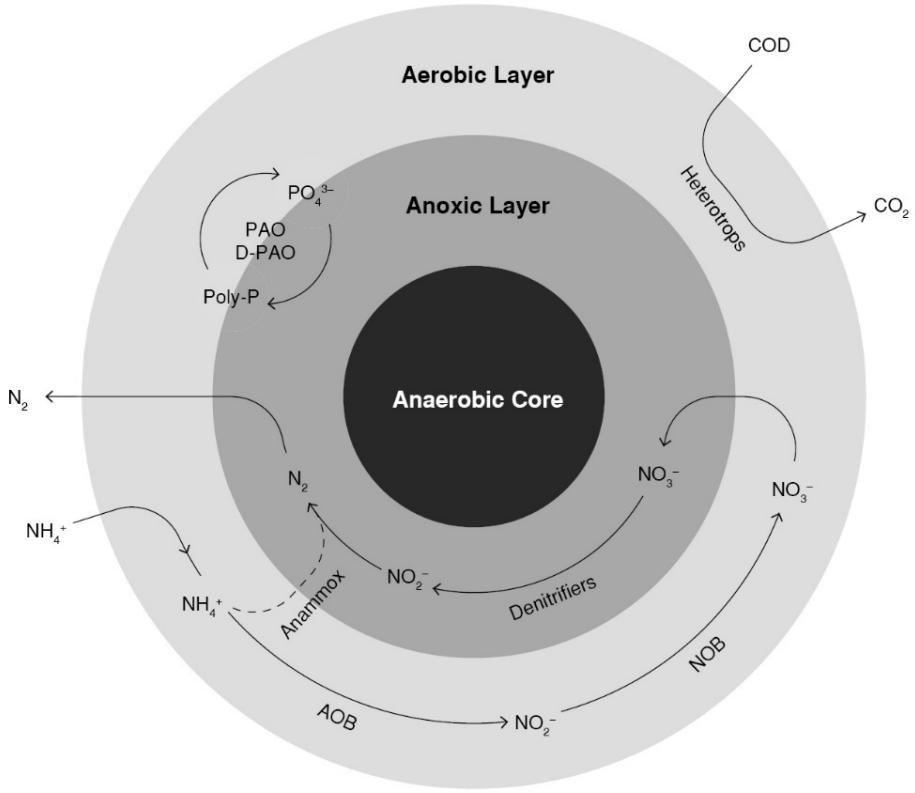

Figure 1: Schematic representation of the structure of an aerobic granule and the biological processes occurring inside.

The term aerobic granular sludge (AGS) originates from the first systems operated entirely at aerobic conditions, while nowadays aerobic, anoxic, and anaerobic conditions are applied to remove more efficiently organic matter and nutrients (Wilén et al., 2018). The cultivation of granules is performed using sequencing batch reactors (SBRs), which operate in consecutive cycles consisting of distinct stages operating in sequence in the same vessel: (1) filling, (2) reaction, (3) settling and (4) withdrawal. Granulation is achieved under certain environmental operations, namely feast-famine feeding, high hydrodynamic shear forces provided by high upflow gas rates, large height to diameter ratio of the reactor, and short settling time to wash-out slow settling microorganisms.

The advantages of this technology compared to the traditional activated sludge process are several: (i) denser structure which excellent settling capacity; (ii) higher biomass concentration; (iii) capability of simultaneous chemical oxygen demand (COD), nitrogen and phosphate removal; (iv) high microbial diversity due to the presence of different microhabitats; (v) longer sludge age; and (vi) the ability to withstand shock loads (Show et al., 2012). Those characteristics allow for a reactor with reduced footprint and cost, which makes AGS an attractive biological treatment technology (Carucci et al., 2010). The features of AGS, together with the good biosorption properties and the higher resistance to toxicity, make this technique also extremely promising for the removal of OMPs (Adav et al., 2008b; Tay et al., 2005). Moreover, the flexibility of operational conditions of the SBR reactor, which allows to adjust the length of different phases and redox conditions, can be an advantage when optimizing the system towards the removal of trace organic pollutants. More information about the impact of process configurations in the removal of OMPs can be found in paragraph 1.2.3.

1.2. Fate of organic micropollutants in biological treatment process

A large number of emerging OMPs have been detected in several different environments (Figure 2). In the aquatic environment, pharmaceutical compounds are one of the most frequently detected classes of OMPs (Rivera-Utrilla et al., 2013). When looking at OMPs such as pharmaceuticals and personal care products, common points of entry are domestic, hospital and manufacturer wastewaters, and leachates from landfill sites. Not being designed for the removal of trace organic contaminants, WWTPs do not remove them successfully and a significant number of hazardous pollutants are released into the receiving waters with the effluent (Luo et al., 2014; Oberoi et al., 2019). Residual concentrations can spread to surface waters, groundwater or sediments. When sewage sludge is used as soil fertilizer, OMPs can spread onto agricultural land and reach the groundwater (Kümmerer, 2009). Veterinary pharmaceuticals directly enter the surface water or though the manure of treated livestock (Le Page et al., 2017).

When advanced physicochemical treatments are not implemented, the effectiveness of the biological treatment units determines the performance of the plant in eliminating those substances. Yet, the mechanisms of removal, the efficacy of the biological units in mineralizing the compounds and the toxicity of OMPs over the microbial communities are still poorly understood and the predictive capacity regarding the ability to remove specific trace organic contaminants remains very limited (Oberoi et al., 2019; Rivera-Utrilla et al., 2013; Tadkaew et al., 2011).

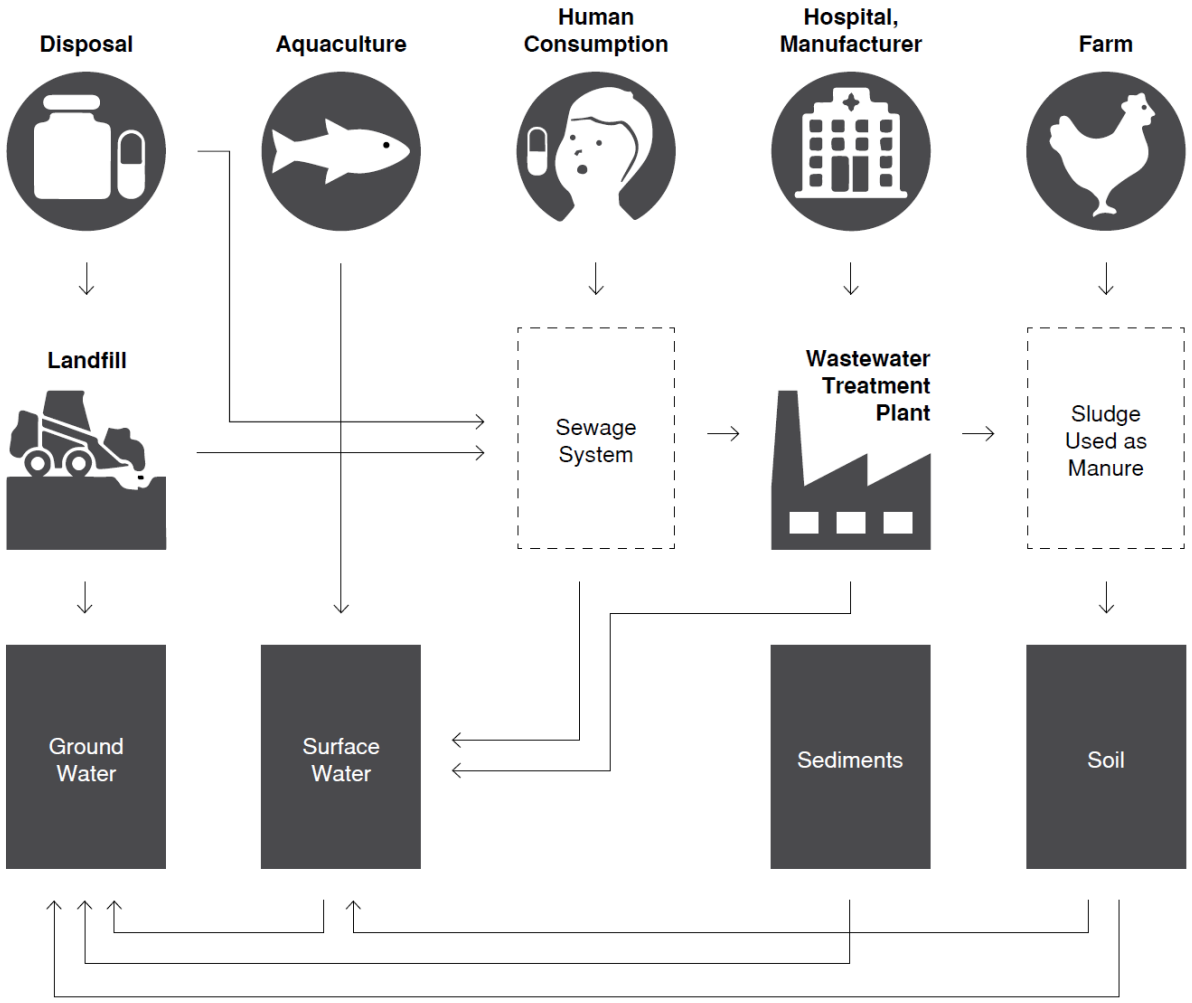

Figure 2: Common points of entry of pharmaceuticals and personal care products and routes into the environment.

1.2.1. **Molecular characteristics of micropollutants**

Significant variation in the removal of OMPs have been reported, ranging from complete elimination of some compounds (i.e. ibuprofen) to almost no removal of others (i.e. carbamazepine, diclofenac). Physicochemical properties of OMPs significantly govern the efficacy of removal in biological processes. These properties are related to the molecular structure: molar mass, availability of functional groups, branching, and ring structure (Tadkaew et al., 2011). Being chemically complex molecules, the molecular weight, structure, functionality, and shape of OMPs vary widely (Rivera-Utrilla et al., 2013). The molecular structure controls the chemical and biochemical reactions the compound might undergo in a biological reactor. It is commonly accepted that the persistency of an organic pollutant is related to the presence of halogens in the structure, nitroaromatic groups, highly branched molecules, while the biodegradability increases with compounds presenting straight aliphatic chains, esters, acids, hydroxyl functional groups (Howard and Muir, 2010). The polarity of the molecules also influences the fate of the compound and its hydrophobicity, hence the tendency to favor a nonaqueous over an aqueous environment. Octanol–water is a reference system that

provides a commonly recognized hydrophobicity measure: the logarithm of the partition coefficient, Log P (or $LogK_{ow}$). Many OMPs are electrolytes and comprise acids, bases, ampholytes, and salt. Compared to neutral molecules, ionogenic compounds have unique characteristics (Trapp et al., 2010): (i) they occur in at least two species (neutral and ionic), whose concentration ratio is dependent on pH; (ii) while neutral compounds are unaffected by electrical fields, ions are attracted or repelled by it; (iii) ions are more polar than the corresponding neutral molecule. For ionic compounds, lipophilicity is not a constant and the pH, which affects the ionization of the molecule, greatly affects octanol-water partitioning. LogD, which is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized), is therefore a more appropriate descriptor for the hydrophobicity of ionizable OMPs.

1.2.2. **Removal mechanisms of micropollutants**

In biological wastewater treatment processes, the removal of OMPs from the aqueous phase is driven by two main mechanisms: sorption onto biomass and biodegradation (Figure 3). While biodegradation transforms the compound, sorption results in a phase transfer. Non-biodegradable substances with high sorption potential can be released into the environment not only with the effluent of the treatment plant, but also with the disposal of the excess sludge on agricultural land (Radjenović et al., 2009a). Sorption to biosolids results in a longer residence time in the reactor, which may lead to further removal via biodegradation. Other minor OMP removal processes in biological wastewater treatment processes involve chemical and physical mechanisms, such as abiotic transformation and volatilization.

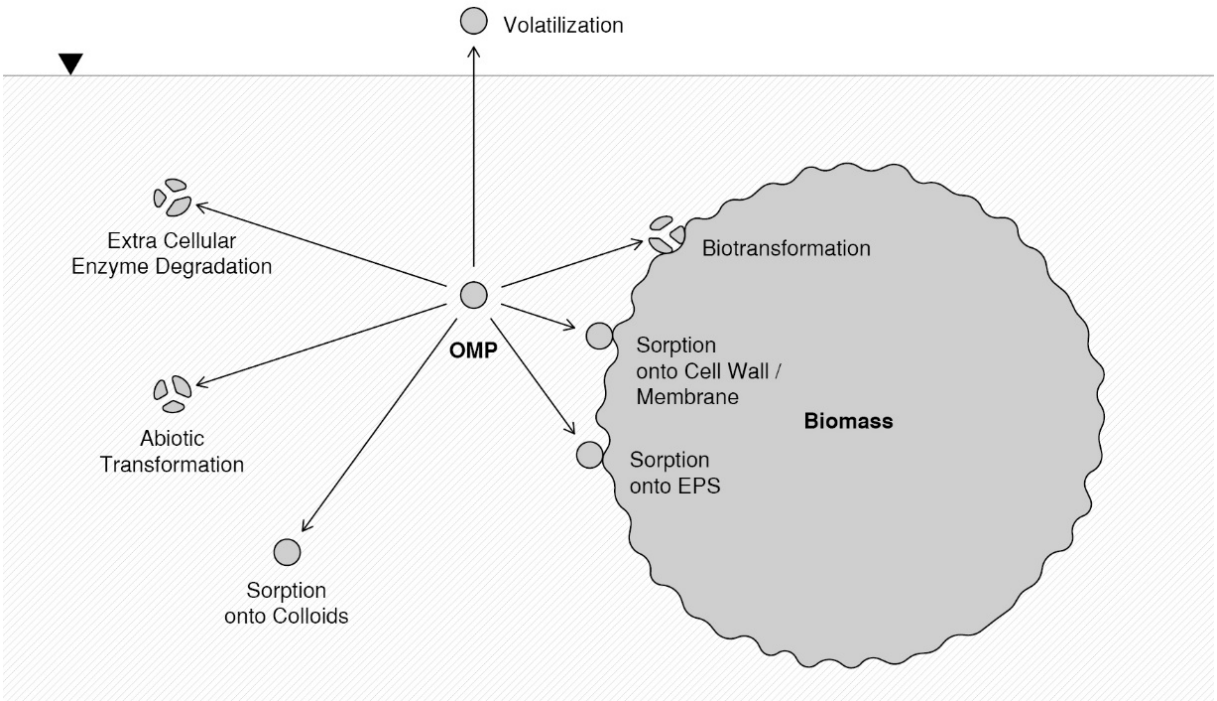

Figure 3: Removal mechanisms of OMPs from the aqueous phase in biological wastewater treatment processes.

Sorption onto biomass

Sorption onto the biomass (or biosorption) can be defined as a metabolically passive physicochemical process involved in the binding of ions or molecules from aqueous solution onto the surface of a sorbent of biological origin. The degree of distribution of the micropollutant molecules in the water phase and in the solid matrix, namely partitioning, is governed by the potential interactions between the compound and the surface of the biomass. The nature of the intermolecular interactions could be hydrophobic and/or electrostatic (Figure 4), including mechanisms such as van der Waals interactions, hydrogen binding, electron donor and acceptor interactions, electrostatic interactions of cations and anions with charged sites of the biomass, cation exchange, cation bridging and ligand exchange with surface bound -OH groups on metal oxides surface (Hyland et al., 2012; MacKay and Vasudevan, 2012; Oberoi et al., 2019; Trapp et al., 2010). Hydrophobic partitioning is important for non-polar molecules and results from the high energy required for a cavity formation in water coupled with the favorable van der Waals interactions between a sorbate and the non-polar domain of the sorbent (Goss and Schwarzenbach, 2003). Electron donor−acceptor complexes involve the attraction of an electron-rich (or -poor) domain on a polar moiety of a neutral sorbate to the complementary electron-poor (or -rich) region on the biomass. Hydrogen bonding belongs to this group of interactions. Ion exchange and cation bridging occur due to the attraction between a charged group of a molecule and oppositely charged moieties on the biomass and are particularly important for ionizable compounds (Polesel et al., 2015). Surface complexation happens when a hydroxyl group or a water molecule bound to a surface metal ion is exchanged with a ligand group on the organic compound (MacKay and Vasudevan, 2012). The OMP hydrophobicity and ionic state are expected to determine which mechanisms of sorption is the predominant one (Rybacka and Andersson, 2016). The liquid media characteristics, in terms of pH and ionic strength, influences the surface chemistry of the sorbent and the activity of the sorbate, and therefore plays a role in the sorption dynamics (Trapp et al., 2010).

Since these interactions happen at specific receptor sites distributed across the biomass solid phase components and involve multiple sorbate functional groups, the characteristics of the solid matrix in the biological reactor (surface charge, specific surface area, EPS content, mineral content, oxidation degree of organic matter) is also determining the degree of partitioning (Alvarino et al., 2018; Barret et al., 2010a; Zhang et al., 2018). As biomass is generally considered negatively charged at the surface, positively ionized micropollutants are likely to show the highest potential for sorption, due to electrical attraction (Franco and Trapp, 2008). The lipophilic cell membrane and cell wall of the microorganisms and the lipid fraction of the sludge are expected to attract hydrophobic compounds (Verlicchi et al., 2012). Different sorption capacity is hence observed with different types of biomass (primary and secondary sludge, digested sludge, biofilms, flocs, anaerobic sludge, etc.). Sorption phenomena also occur onto the colloidal matter in the aqueous phase (Holbrook et al., 2004) and it is likely to influence the sorption to particles (Barret et al., 2010b). The partitioning of OMPs between the aqueous phase and the biomass is generally assumed instantaneous (Pomiès et al., 2013) and involves two reverse mechanisms: sorption from aqueous to solid phase and desorption from solid to aqueous phase (Joss et al., 2006). The equilibrium is reached when the rate of sorption equalizes the rate of desorption.

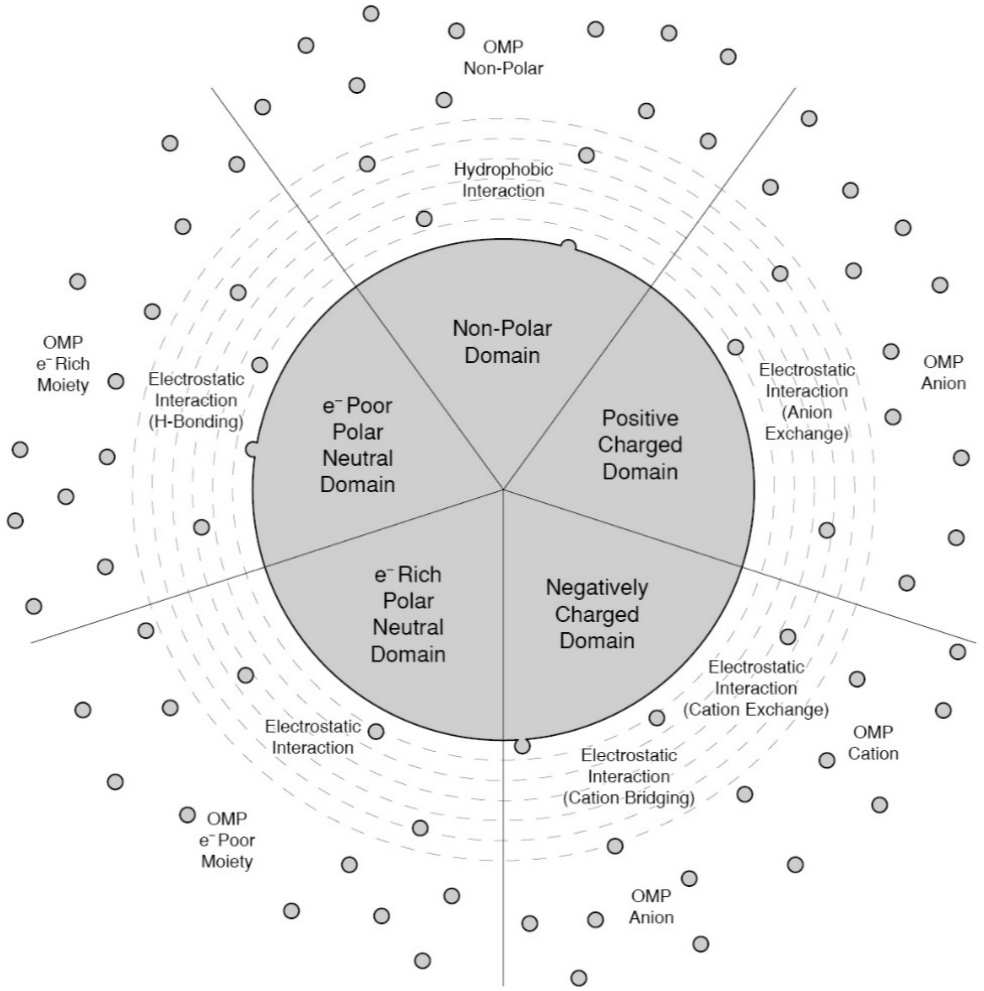

Figure 4: Schematic of the sorption of OMPs onto distinct receptor sites of the biomass via multiple interaction mechanisms.

Biotransformation

Microbial biotransformation refers to the microbially mediated conversion of a parent compound to a transformed product called metabolite or metabolic intermediate. Different intermediates can be formed by either the breakdown of the parent compound or its modification (Oberoi et al., 2019). Those metabolic reactions can be intracellular or extracellular (Fischer and Majewsky, 2014; Stadlmair et al., 2018). Biotransformation is considered a major pathway for the neutralization of OMPs, even though it is still unknown whether the compounds are fully mineralized or transformed into metabolites that might be even more environmentally problematic than the parent molecule, especially when biologically active moiety remains intact during degradation (Boxall et al., 2004; Magdeburg et al., 2014). Microbial transformation can be driven metabolically or co-metabolically (Stadlmair et al., 2018). Being present at extremely low concentrations that could not sustain growth, OMPs are supposed to be transformed dominantly by cometabolic processes, i.e. are biodegraded by non-specific enzymes generated by the primary substrate metabolism (Fischer and Majewsky, 2014). These enzymes are acting as catalyzers to lower the activation energy for a reaction to occur and their production is induced by the presence of a primary substrate which provides

carbon and energy for the microorganisms (Illanes, 2008). An example of a relatively unspecific enzyme that has been reported to co-oxidize OMPs, is AMO (Men et al., 2017). The extent of transformation of the parent compound depends on the degree of structural analogy between the OMPs and the primary substrate (Rieger et al., 2002). The electron withdrawing and donating groups in the molecule are therefore of importance. Electron withdrawing groups render the OMP less susceptible to the electrophilic attack generated by mono- or dioxygenases of aerobic bacteria. Theoretically, OMPs with electron withdrawing groups should be more readily degraded by a reductive or nucleophilic rather than by an oxidative or electrophilic mechanism (Rattier et al., 2014). Electron donating functional groups, on the other hand, render the molecules more prone to electrophilic attack by activated oxygen species produced in the oxidative catabolism (Tadkaew et al., 2011; Wei et al., 2019).

Other removal processes

Other OMP removal processes in biological wastewater treatment processes involve chemical and physical mechanisms. Volatilization results in a phase transfer and it is considered negligible for OMPs having Henry's coefficient $H < 10^{-5}$ those compounds (Schwarzenbach et al., 2003). For volatile compounds or slightly volatile, such as musk fragrances ($H \sim 0.005$), the stripping efficiency of the reactor should be investigated. Chemical removal mechanisms include hydrolysis, chemical oxidation, chemical reduction, and photodegradation. During hydrolysis, intramolecular OMP bonds are broken down by reaction with water and the hydroxyl group is introduced in the molecule, replacing one of the groups of the parent compound. The groups that might be susceptible to hydrolysis are amides, carboxylic acid esters, lactones, phosphoric acid esters (Neely and Blau, 1985). Chemical oxidation and reduction, which involves the transfer of electrons from the reduced to the oxidized species, have been observed, for example the oxidation of halogenated solvent and the reductive dehalogenation of halogenated compounds (Kalavrouziotis, 2017). Photodegradation occurs in the presence of light and it involves the absorption of a photon by a compound and the formation of excited or radical species with a modified chemical structure compared to the parent compound. This process might not be relevant in reactors where the penetration of light is minimal, but it is an important removal mechanism in exposed biological systems such as constructed wetlands (Zhang et al., 2014). Other abiotic transformation processes include epimerization, nitration and nitrosation (Polesel et al., 2016).

1.2.3. Impact of process configuration on removal efficiency

The efficiency of biological reactor to degrade OMPs is greatly determined by its operational conditions (Kang et al., 2018; Su et al., 2015). The **hydraulic residence time (HRT)**, corresponding to the average residence time of the liquid phase in the reactor, affects the contact time between the biomass and the OMP. Considering the general slow removal rate for OMPs, a longer HRT might be beneficial and has been observed to positively influence the removal of several OMPs (Gros et al., 2010). Longer HRT usually corresponds to increased reactor volume and footprint, which might not be feasible at a full-scale treatment plant. The sludge retention time, indicated also as **solid retention time (SRT),** designates the mean residence time of microorganisms in the reactor and is related to the growth rate of microorganisms. The role of SRT has been widely studied and discussed (Grandclément et al., 2017; Pomiès et al., 2013; Tiwari et al., 2017). The positive influence on OMP removal at longer SRT is generally credited to the higher microbial diversity, which increases the number of possible metabolic pathways (Falås et al., 2016). High SRT encourages the growth of slow-

growing organisms such as nitrifiers, which seem to cometabolize a wide group of OMPs via AMO (Park et al., 2017). The **temperature** in the biological reactor affects the microbial activity and even the solubility of OMPs, but inconclusive observations have been reported so far (Tran et al., 2013). Temperature has also been observed to influence sorption of several OMPs onto biomass (Xu et al., 2008; Zeng et al., 2009). The **pH** of the liquid phase influences the microbial community (Tran et al., 2013) and determines the protonation state of the compounds, affecting its solubility and the sorption capacity onto the biomass (Trapp et al., 2010). The **turbulence** in the reactor seems to affect sorption of lipophilic compounds as it affects the mass transfer from the liquid to the solid phase (Alvarino et al., 2014).

The **redox conditions** can enhance the biotransformation of a specific OMP rather than another. The redox conditions also govern the microbial community in a biological reactor and therefore the enzyme produced. Some compounds, i.e. atenolol, naproxen, have been observed to transform under both aerobic and anaerobic conditions, while others, such as venlafaxine, sulfamethoxazole, were removed better under anaerobic conditions (Alvarino et al., 2018; Falås et al., 2016; Stadler et al., 2015). Aerobic transformation seems to be predominant compared to anaerobic biodegradation (Alvarino et al., 2014; Tran et al., 2013). A combination of different redox conditions has been shown to broaden the group of OMP that are degraded (Suarez et al., 2010). **Food to microorganism** ratio is likely to have an effect on the microbial activity. A lower ratio translates in a shortage of biodegradable organic matter, which could stimulate the metabolization of more persistent compounds (Park et al., 2017). The **primary growth substrate** can influence largely the cometabolic degradation of trace organic pollutants (Çeçen and Tezel, 2017). It has been observed to suppress the biotransformation rate of OMPs (Su et al., 2015) and to promote the metabolic capability of the microbial community by acting as microbial selector (Li et al., 2014).

High **biomass concentration** increases the biological activity of microorganisms, hence enzymatic activity, leading to greater OMP biotransformation potential (Park et al., 2017; Radjenović et al., 2009b; Yu et al., 2009). The **biomass configuration**, biofilm or floccular, could affect the performances of the biological reactor. In a biofilm system, the microorganisms being embedded in a matrix of EPS benefit from a protection layer from inhibiting or hazardous substances (Schmidt et al., 2012). It is unlikely that OMPs at the concentrations found normally at the WWTP should influence nutrient removal performances. On the other hand, it has been shown that the presence of OMPs enhances EPS production (Pasquini et al., 2013). Higher OMP removal rates have been observed in biofilm carriers compared to activated sludge (Falås et al., 2012). The composition of **EPS**, being a complex mixture of polymers, offer potential binding sites due to presence of anionic, cationic and apolar functional groups (Wunder et al., 2011; Zhang et al., 2018). EPS have been observed to be one of the key components in the sorption removal of OMPs (Xu et al., 2013) and their composition in terms of protein and polysaccharides content to influence the degree of it (Khunjar and Love, 2011). **Biofilm thickness**, as a result of diffusion limitation and thus substrate penetration, is expected to have an impact on micropollutant removal. Thicker biofilm has been shown to increase microbial diversity and biotransformation kinetics of several OMPs (Torresi et al., 2016). Also, the size of the biomass has an impact on sorption potential. Since smaller particles offer a larger specific surface area, sorption is enhanced as the size decreases (Alvarino et al., 2015).

2. Research motivation and scope of the thesis

Conventional WWTPs have been identified as a point source of emission of OMPs to the aqueous environment. Not being designed for the elimination of trace organic contaminants, the removal is often incomplete, resulting in continuous discharge. Advanced treatment processes such as adsorption onto activated carbon, oxidation by ozone and membrane systems have been found efficient for removal of a wide range of OMPs (Rizzo et al., 2019). However, these techniques have high energy consumption and negative environmental impact. Biological treatments might offer a lower carbon footprint compared to advanced treatment processes. Among biological processes, biofilm displays advantages in removing OMPs. The longer solid retention time, the higher microbial diversity and the presence of micro-niches of different redox conditions are features of biofilm that make this system very attractive for the removal of OMPs. As the mechanisms of removal, the efficacy of mineralization of compounds by biological units, and toxicity of OMPs over microbial communities are poorly understood, the predictive capacity for removing specific trace organic contaminants remains very limited. AGS is a novel technique where the microorganisms grow in dense aggregates with excellent settling, higher biomass retention and capability of COD, N and P removal, characteristics that enable small footprint and flexible mode of operation. This form of free-floating biofilm harbors a very diverse microbial community and have been found promising for the removal of toxic compounds. The characteristics of AGS, together with the good biosorption properties, the high biomass concentration and the possibility of operating at different redox conditions, make this technology especially suitable for the removal of micropollutants. The elimination ability of OMPs by AGS is largely unknown and the advantages of this biofilm process have still to be elucidated.

The overall goal of the research project is to study the elimination mechanisms of OMPs in biological treatment and the removal potential of biofilm processes, from both full-scale treatment plants and laboratory reactors, with focus on AGS. Due to the significance for municipal wastewater contamination, focus is laid on pharmaceuticals.

To fulfill the goal several specific objectives are included in this thesis. The objectives of this work are:

- To determine which OMPs can be removed by sorption onto AGS (Paper I);
- To compare the sorptive capacity of different conformation of AGS (Paper I);
- To study OMP characteristics and correlate them to the sorption removal in AGS (Paper I);
- To investigate the potential of different biofilm systems at the full scale WWTP to eliminate OMPs (Paper II);
- To develop well-controlled laboratory scale AGS SBR to study the treatment performances, OMP exposure during long term operations and bacterial community structure.

To accomplish this, research on this topic was conducted and the results are presented as one manuscript (I) and one conference paper (II).

In paper I, the biosorption capacity of aerobic granular sludge was investigated. This study compares the sorption removal of 22 micropollutants by two types of aerobic granular sludge, one dominated by fungi and one dominated by bacteria, at different granule sizes.

In paper II, the presence and removal of targeted trace organic contaminants in a large Swedish WTTP is presented. Release of OMPs has been found to be significantly influenced by the biological treatment processes. In a WWTP consisting of a combination of suspended- and attached growth reactors with different redox conditions, it is unclear which process contributes the most to OMP removal. A total of 25 pharmaceutical substances, two insecticides and a plasticizer, bisphenol A (BPA), were measured in the wastewater influent and effluent of the different biological treatment processes, which include activated sludge reactor, nitrifying trickling filters, nitrifying MBBRs and post-denitrifying MBBRs.

3. Materials and methods

3.1. Experimental design

3.1.1. Optimization of the lab scale AGS design

To study the treatment performances, OMP exposure during long term operations and bacterial community structure of AGS exposed to OMPs, well-controlled laboratory scale systems were necessary for this type of investigation. A new lab scale reactor was assembled and optimized to have controlled redox conditions, pH and temperature, which are factors that can significantly affect OMP removal performances. The operational conditions in terms of design of the different phases, cycle length, settling strategy and synthetic wastewater were also adjusted to promote nitrification/denitrification and biological phosphorus removal and to cultivate granules with a diverse microbial community structure. The components of the new reactor were assembled, calibrated and adjusted to deliver stable operations of the reactor over a period of three months. During this time, nine selected OMPs were dosed in the influent of the SBR at a concentration of 10 µg/L to study the removal performances of the reactor and the effect of OMP exposure on nutrient removal efficiency.

3.1.2. Biosorption of selected OMPs onto granules (Paper I)

To investigate which OMPs can be removed from the water phase by sorption onto granules and to evaluate the extent of removal by sorption, batch experiments were performed with 22 pharmaceutical compounds exposed to two types of aerobic granular sludge, one dominated by fungi-like structure and one dominated by bacteria-like structure. Batch sorption experiments were performed with inactivated granules of controlled thickness (diameters of 0.5-1, 1-2, and >2 mm) and initial micropollutant concentration of 100 µg/L.

Table 1: biological samples identification for the sorption tests

Sieve opening	0.5-1 mm	1-2 mm	>2 mm
Bacteria dominated	BS	BM	BL
Fungi dominated	FS	FM	FL

3.1.3. Removal of organic micropollutants in the biological units of a Swedish wastewater treatment plant (Paper II)

The presence and removal of target OMPs were investigated in a large Swedish wastewater treatment plant designed for nutrient removal including a series of different biological reactors, including activated sludge, trickling filters, nitrifying moving bed biofilm reactors (MBBRs) and post-denitrifying MBBRs. A total of 28 organic micropollutants was analyzed, at concentrations ranging from few ng/L to µg/L, in the influent and effluent of the different biological reactors in two sampling campaigns.

3.2. Experimental set-up

3.2.1. Reactor design for the cultivation of granules

Two identical lab-scale SBRs were employed for the cultivation of granules for the biosorption experiments (Figure *5*). The plexiglass vessels were column shaped open reactor with a 6 cm diameter, 132 of total height and a height of 110 cm of the water level, resulting in a working volume of 3 L. The effluent port was located at 63 cm from the bottom corresponding to a volume exchange ratio of 43%. The feed was pumped from the bottom of the vessel. Compressed air was provided with a stone diffuser with a pore class I (100-160 µm pore size). The reactors were operated in a four-hour cycle, comprising 60 min of anoxic feeding, 173 min of aeration, 2 min of settling, 5 min of sludge withdrawal. The settling time was gradually decreased during the operation to allow a better retention of nitrifying organisms (Szabó et al., 2016) and the aerobic reaction phase was adjusted consequently to reach a total 4 hours cycle length. Aerobic conditions were maintained by sparging compressed air at a flow of 2.5 L min^{-1} and superficial up-flow velocity of 1.5 cm s^{-1}. The DO and pH in the reactor were not controlled. The pH was monitored, and the data logged into a computer. The DO was monitored over a series of cycles and revealed oversaturated conditions during the aeration time. The temperature was ambient approximately 20 ± 3 °C.

Wastewater composition and sludge inoculum

A mineral composition with a single organic carbon source was used with a load of 2 kg COD $m^{-3}d^{-1}$, 0.3 kg NH^{4+}-N $m^{-3}d^{-1}$, and 0.1 kg PO^{4-}-P $m^{-3}d^{-1}$ as described previously (Liébana, 2019). The synthetic wastewater consisted of 994.2 mg L^{-1} $NaCH_3COO$, 443.8 mg L^{-1} NH_4Cl, 12.5 mg L^{-1} $MgSO_4 \cdot 7H2O$, 15.0 mg L^{-1} $CaCl_2$, 10.0 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 139.5 mg L^{-1} KH_2PO_4, 56.5 mg L^{-1} K_2HPO_4. Micronutrient solution contained 0.05 g $L^{-1}H_3BO_3$, 0.05 g $L^{-1}ZnCl_2$, 0.03 g $L^{-1}CuCl_2$, 0.05 g $L^{-1}MnSO_4.H_2O$, 0.05 g $L^{-1}(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 g $L^{-1}AlCl_3$, 0.05 g $L^{-1}CoCl_2.6H_2O$, and 0.05 g $L^{-1}NiCl_2$) and 1 mL L^{-1} was added (as described in (Tay et al., 2001). The feed was prepared in three separated containers, one for the concentrated carbon source, one for the phosphorus and nitrogen species and one with the micronutrients. The reactors were operated with the same conditions, with the only difference in the carbon source used, being for one reactor (R1) sodium acetate and for the second (R2) sodium acetate and acetic acid (50-50 COD ratio). The reactors were inoculated with activated sludge from a full scale WWTP located in Tanum (Sweden). Fresh sludge was collected and introduced in the reactors the following day. The sludge was concentrated two-fold by letting the first batch to settle, removing the supernatant and refilling the vessels with a second batch of sludge.

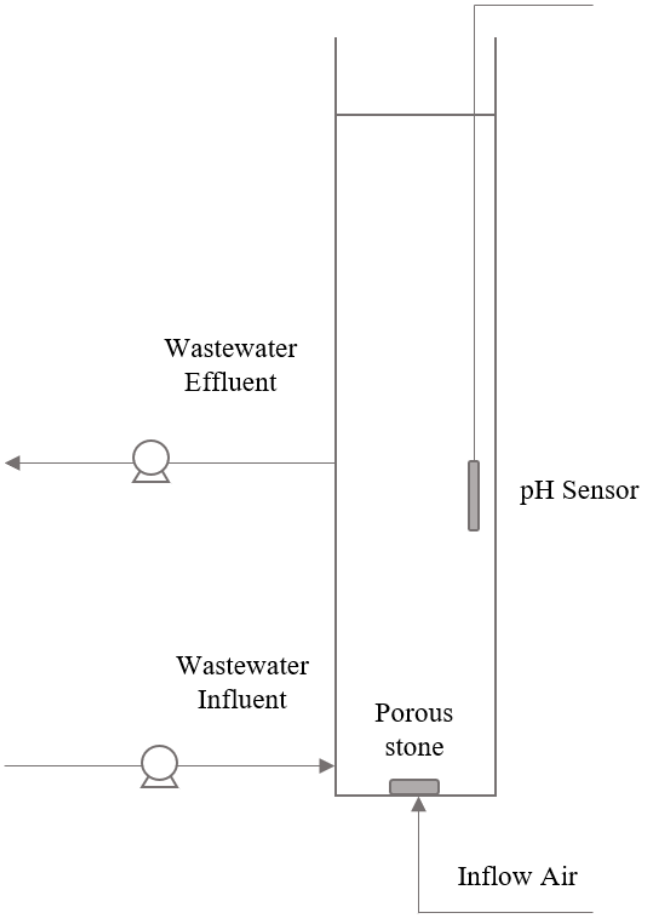

Figure 5: Schematic representation of the reactors used to cultivate granules (non-scaled image).

3.2.2. **Reactor design for the removal of OMPs**

The schematic of the lab scale AGS reactor assembled for the long-term operation experiments is illustrated in Figure 6. To minimize abiotic losses due to sorption onto the reactor material, the bioreactor had to be constructed in glass. The vessel was purchased from LouwersHanique B.V. (The Netherlands), and had a 5.6 cm diameter, 160 cm of total height and a double wall for possible temperature control with a thermostat bath. To allow the operation under different aerobic, anoxic and anaerobic conditions, the new design had to be a close system to avoid the intrusion of oxygen and to permit the monitoring of reactions products such as CO_2, CH_4, N_2, NO_x. A gas recirculation system was installed to control the oxygen saturation inside the reactor at levels lower than 100%. The return gas was conveyed into a vessel connected to the two Mass Flow Controllers (MFC, Bronkhorst) injecting N_2 gas or air, depending on the desired oxygen saturation level. A diaphragm gas pump (KNF) conveyed the mixed gas to the bottom of the reactor. The gas flow was controlled at 2.5 L min^{-1} by a rotameter and was supplied through a metal sparger (1-2 µm pore size). The pH value was controlled at 7.0 ± 0.3 by addition of acid and base solutions (HCl and NaOH). A National instrument data acquisition hardware was used to monitor and record the signals from the DO, pH and pressure sensors. A control unit (Omniprocess AB) operated the reactor components (peristaltic pumps, MFCs, gas pump). Reactor cycles had a length of 360 minutes, consisting of 90 minutes anaerobic feeding,

257 minutes aeration, 3 minutes settling, and 10 minutes effluent withdrawal. The settling time was gradually decreased during the operation and the aeration phase adjusted accordingly for a total cycle time of 6 hours. The hydraulic retention time was 12 hours and the volumetric exchange ratio per cycle was 50 %. The DO level was maintained at 20 ± 8 % saturation during the aeration phase. The temperature of the reactor was maintained at 21 ± 0.5 °C in a temperature-controlled room protected by sunlight.

Wastewater composition and sludge inoculum

A mineral composition with multiple organic carbon sources was used with a slight modification of the recipe used by Layer et al. (2019). Multiple substrate degradation has been associated to larger microbial richness (Mery-Araya et al., 2019) and a more complex synthetic media has been observed to be a surrogate closer to real municipal WW, compared to a carbon feed made exclusively of VFAs (Layer et al., 2019). Acetate, propionate, glucose and peptone from enzymatic digest provided the same amounts of COD equivalents. The synthetic wastewater consisted of 192.3 mg L^{-1} $NaCH_3COO$, 120.1 mg L^{-1} CH_3CH_2COONa, 140.8 mg L^{-1} $C_6H_{12}O_6$, 122 mg L^{-1} peptone from enzymatic digest, 90.6 mg L^{-1} NH_4Cl, 16.3 mg L^{-1} $MgSO_4 \cdot 7H2O$, 20.1 mg L^{-1} $CaCl_2$, 32.8 mg L^{-1} KCl, 13.2 mg L^{-1} KH_2PO_4, 16.9 mg L^{-1} K_2HPO_4. Micronutrient solution contained 0.05 g $L^{-1}H_3BO_3$, 0.05 g $L^{-1}ZnCl_2$, 0.03 g $L^{-1}CuCl_2$, 0.05 g $L^{-1}MnSO_4.H_2O$, 0.05 g $L^{-1}(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 g $L^{-1}AlCl_3$, 0.05 g $L^{-1}CoCl_2.6H_2O$, and 0.05 g $L^{-1}NiCl_2$) and 1 mLL^{-1} was added (as described in (Tay et al., 2001). The mineral composition provided a total carbon:nitrogen:phosphorus ratio of approx. 100:7:1 and a load of 1.20 kg COD $m^{-3}d^{-1}$, 0.08 kg NH^{4+}-N $m^{-3}d^{-1}$, 0.01 PO^{4-}-P $m^{-3}d^{-1}$. The nitrogen and carbonaceous species were prepared in 20-fold concentration, while the phosphorus and micronutrients in 100-fold concentration. The reactor was inoculated with activated sludge from a full scale WWTP located in Kungsbacka (Sweden). Fresh sludge was collected and introduced in the reactor the same day. The sludge was concentrated two-fold.

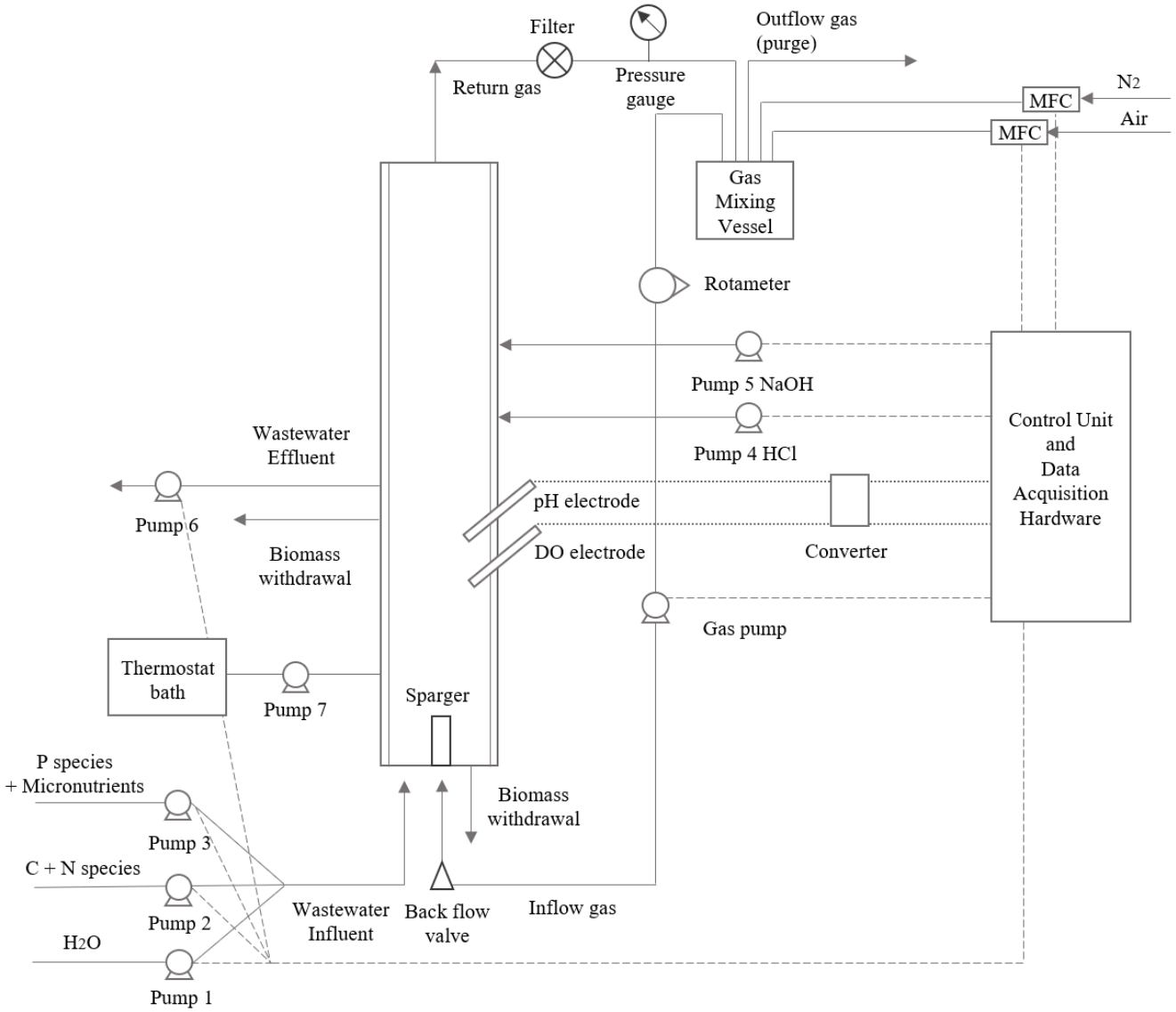

Figure 6: Schematic setup of the new AGS reactor assembled for the long-term operations of OMP removal (non-scaled image).

3.2.3. **OMP sorption batch experiments**

The sorption of OMPs onto granular sludge was studied by exposing a group of pharmaceutical compounds at concentration around 100 µg L^{-1} to inhibited biomass. Details about the batch sorption test can be found in Paper I. Briefly, the biomass was extracted from the reactor, rinsed with phosphate buffer and inhibited with 0.2 g NaN_3/gTSS. A mix of OMPs dissolved in a mineral recipe were exposed to the inhibited biomass and at regular intervals mixed samples were extracted to measure the dissolved concentration of OMPs. The batch experiments were performed in 300-mL glass beakers in a water bath, at ambient temperature (19 ± 0.5°C) and pH equal to 7.0 ± 0.3, controlled by dosing NaOH and HCl 1M. Mixing in the beaker was provided by sparging of nitrogen at a controlled flow of 2 L min^{-1} to guarantee a constant level of turbulence. The experiment duration was set to 6 hours. In activated sludge system, the solid-water equilibrium can be achieved within 0.5-1h (Ternes et al., 2004), while in granules it has been shown that maximum sorption capacity is reached after 4 hours (Shi et al., 2011). Other abiotic removal processes, such as volatilization, sorption on glass wall and lab ware were assessed in a negative control test, where pharmaceuticals were spiked in the same media, including the biocide but without biomass.

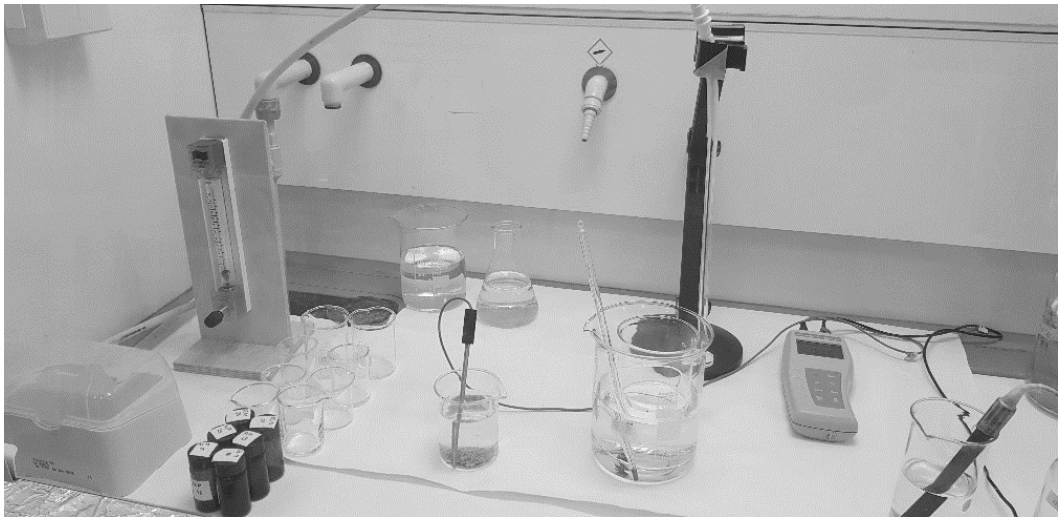

Figure 7: Picture of the batch experiments with a schematic representation of the components and chemicals used.

At equilibrium, the OMP concentration sorbed onto the biomass ($C_{s,eq}$ µg L^{-1}) is proportional to the dissolved concentration ($C_{aq,eq}$, µg L^{-1}) and the solids-water distribution coefficient (K_d) can be expressed as (Equation 1) (Torresi et al., 2017):

$$K_d = \frac{C_{s,eq}}{C_{aq,eq} X_{biomass}} = \frac{C_{aq,0} - C_{aq,eq}}{C_{aq,eq} X_{biomass}} \quad (1)$$

The concentration sorbed onto the biomass ($C_{s,eq}$ µg L^{-1}) can be estimated from the initial OMP concentration ($C_{aq,0}$ µg L^{-1}) and the measured final concentration at equilibrium ($C_{aq,eq}$ µg L^{-1}). This linear equation is a simplified case of the empirical Freundlich isotherm (Schwarzenbach et al., 2003).

3.2.4. **OMP selection**

The OMP selection was dependent on the laboratory technique available for the analyses. The UPLC MS/MS technique at Kristianstad University could detect and quantify a group of 28 OMPs. The compounds differ widely in structure and behavior and are classified according to their use (Table *2*). For the biosorption test, a selection of those compounds was made with focus on pharmaceutical substances. Stock solutions of individual compounds (100 mg L^{-1}) were prepared in ethanol (70%) and were stored in amber glass bottles at 4 °C. The substances were obtained from Sigma Aldrich (Steinheim, Germany).

Table 2: the OMPs targeted in the full-scale study and the selection for the batch sorption experiments.

Class	**Compound**	**Abbreviation**	**Biosorption test**	**AGS SBR**
Antibiotics	Sulfamethoxazole	SMX	X	X
	Trimethoprim	TMP	X	
	Ciprofloxacin	CIP	X	X
	Clarithromycin	CLY	X	
	Erythromycin	ERY	X	
Anticonvulsant	Carbamazepine	CBZ	X	X
Antidepressants	Sertraline	SER	X	X
	Citalopram	CTL	X	X
	Venlafaxine	VEN		X
Antifungal	Ketoconazole	KTC	X	
	Fluconazole	FCL	X	
Antihypertensive	Metoprolol	MET	X	X
	Atenolol	ATN		X
	Losartan	LOS	X	
Antineoplastic	Methotrexate	MTX	X	
Diuretic	Furosemide	FUR		
Hormones	β-Estradiol	E2	X	
	17α-Ethinylestradiol	EE2	X	
	Levonorgestrel	LEV	X	
Insecticide	Imidacloprid	IMD		
	Acetamiprid	ACT		
Non-steroidal anti-inflammatory	Diclofenac	DCF	X	X
	Naproxen	NPX	X	
	Ibuprofen	IBP	X	
Opiate analgesic	Tramadol	TRA	X	
Plasticizer	Bisphenol A	BPA		
Sedative hypnotic	Oxazepam	OXA	X	
	Zolpidem	ZOL	X	

3.3. Analytical methods

The reactor performances were investigated by analyzing the dissolved organic carbon, nitrogen and phosphorus content of aqueous samples. Soluble organic carbon and nitrogen was measured with a TOC/total N analyzer (Shimadzu) after filtration through a polyethersulfone membrane syringe filter of 0.2 µm pore size. Cations (NH^{4+}-N) and anions (NO^{3-}-N, NO^{2-}-N, PO^{4-}-P) were analyzed with an Ion Chromatograph (Dionex ICS-900). Samples from micropollutant analysis were analyzed with UPLC MS/MS via direct injection using internal standards. Information about internal standards, UPLC MS/MS technique and methods, limits of detection and quantification are shown in (Svahn and Björklund, 2019).

3.4. Sludge parameters

Physical sludge parameters TSS, VSS and SVI5, SVI10, SVI30 were quantified using standard methods (APHA, 2005). The SRT was calculated according to Equation 2:

$$SRT = \frac{V_r \, TSS_r}{Q_{exc} \, TSS_{exc} + Q_{eff} \, TSS_{eff}} \quad (2)$$

The reactor volume was expressed as V_r (L), TSS_r, TSS_{exc} and TSS_{eff} were the TSS concentrations (gTSS L^{-1}) in the reactor, excess sludge and effluent respectively, Q_{exc} and Q_{eff} were the flow rate (L d^{-1}) of excess sludge and effluent respectively. The sludge size fractions were separated by sieving the sludge at 2-, 1- and 0.5-mm. Sludge morphology was observed by light microscopy (Olympus BX53) equipped with a digital camera (Olympus DP11) on bi-weekly and monthly basis.

3.5. Microbial Adhesion To Solvents (MATS)

The hydrophobic nature of AGS was determined as microbial cell hydrophobicity and electron exchange by the method described by Bellon-Fontaine et al. (1996). Details about the protocol used for granules can be found in Paper I. The biomass was harvested, sonicated to create a cell suspension and the supernatant diluted with phosphate buffer to reach a final absorbance of approximately 0.5 ± 0.1 (Figure *8*). The absorbance was evaluated at 600 nm with a spectrophotometer (UV-1201, Shimadzu). Resuspended cells were then mixed vigorously with 1 mL of the solvent by vortexing and the emulsion was left to stand for 10 min to allow the two phases to separate. The final aqueous phase absorbance at 600 nm was measured spectrophotometrically. The adhesion to each solvent was calculated as a percentage (Equation 3):

$$\% \text{ Adhesion} = 100 \left(1 - \frac{A}{A_0}\right) \quad (3)$$

where A_0 is the absorbance at 600 nm of the suspension before mixing and A is the absorbance of the suspension after mixing with one of the solvents. It should be noted that the method does not determine the hydrophobicity of the surface of the granules, rather it expresses the hydrophobicity of the cell components of granules. Hydrophobicity can vary within the depth of the granules and be higher at the surface of the granules compared to the core as it was found in the study conducted by Wang et al., (2005).

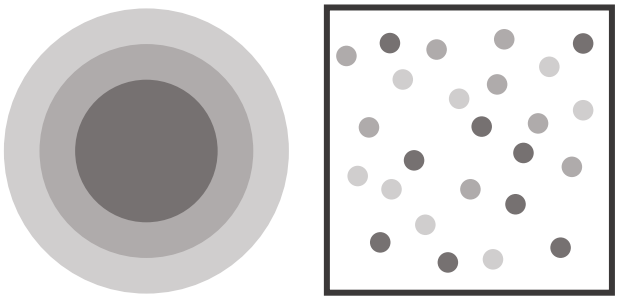

Figure 8: MATS test determines the hydrophobicity of the emulsion of cell components of the granules.

The MATS method is based on comparing microbial cell affinities for monopolar and nonpolar solvents which exhibit similar van der Waals surface tension components. The selected pairs of solvents were the following: (i) chloroform (electron acceptor acidic solvent), and hexadecane (hydrophobic nonpolar n-alkane), and (ii) diethyl-ether (electron donor basic solvent), and hexane (hydrophobic nonpolar n-alkane). In Table *3*, the solvent characteristics in terms of free energy components are described.

Table 3: selected pair of solvents and energy characteristics

Solvent	**Polarity**	**y^{LW} (mJ/m^2)**	**y^{+} (mJ/m^2)**	**y^{-} (mJ/m^2)**
Hexadecane	Apolar	27.7	0	0
Chloroform	Monopolar-acidic	27.2	3.8	0
Hexane	Apolar	18.4	0	0
Diethyl ether	Monopolar-basic	16.7	0	16.4

Two components contribute to the total surface free energy y^{TOT} (Equation 4):

$$y^{TOT} = y^{LW} + y^{AB} \quad (4)$$

y^{LW} is the nonpolar part related to Lifshitz–Van der Waals interactions and y^{AB} is the acid-base constituent which results from the electron-donor (y^{-}) and electron-acceptor (y^{+}) molecular interactions (Lewis acid–base interactions) (Prokopovich and Perni, 2009).

3.6. Rya WWTP

The presence and removal of target OMPs were investigated at the Rya WWTP, which treats wastewater from 970 000 pe in the Gothenburg region (Sweden). The yearly average incoming wastewater flow is 387 470 m^3 d^{-1} with annual average concentration of 349 mg L^{-1} COD_{Cr}, 160 mg L^{-1} BOD_7, 27 mg L^{-1} N_{tot}, 3.4 mg L^{-1} and P_{tot}, 349 mg L^{-1} COD. Wastewater treatment includes primary settling, chemical removal of phosphorus, and biological nitrogen removal. The WWTP consists of several biological treatment processes, including activated sludge reactor for denitrification and organic matter removal, nitrifying trickling filters, nitrifying moving bed bioreactors (MBBRs) and post-denitrifying MBBRs (Figure 9). The sampling was performed over

two separate summer campaigns and grab samples were collected at the influent and effluent of the different biological reactors. During the campaigns, the AS was operated at an average SRT of 6.7 days and a sludge recirculation equal to 0.99 times the inflow. The nitrifying trickling filter flow was 2.17 times the inflow. The average water temperature was 17.4 °C.

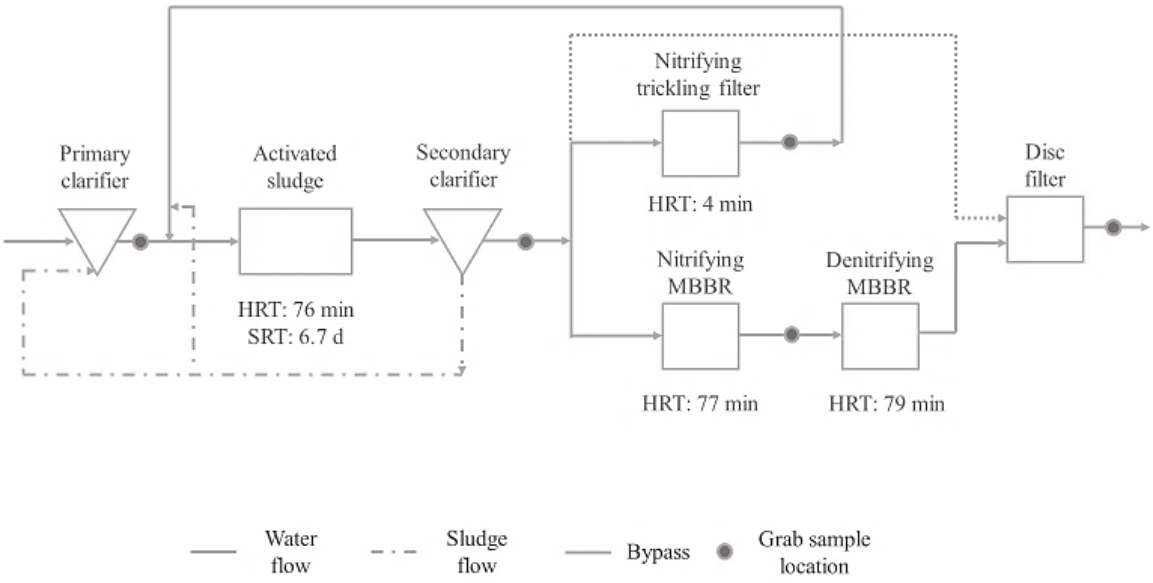

Figure 9: Process scheme and sampling location at the full scale WWTP located in Gothenburg.

4. Results and Discussion

4.1. Performances of the lab scale AGS reactors

4.1.1. AGS reactors for granule cultivation

The two reactors were operated in parallel under the same conditions, except for the source of organic carbon. The mixture of sodium acetate and acetic acid dosed in R2 very likely resulted in different trends of pH compared to R1, which was fed only by sodium acetate. In a typical cycle, the pH varied from 7.9 to 9.3 in R1 and from 5.5 to 9.2 in R2. In R2, filamentous structures started to emerge in the granules from day 90. An acidic pH has previously been observed to cause filamentous overgrowth in AGS (Wan et al., 2014; Xiao et al., 2008). The COD, nitrogen and phosphorus removal efficiencies of the reactors for the period in which sorption batch tests were performed (between day 166 to day 196 from inoculation) are listed in Table 4. While R1 showed good removal performances in terms of nitrogen and COD, the reactor dominated by fungi, R2, exhibited poor performances in oxidizing ammonia. Phosphorus was not removed by R2 and moderately eliminated by R1. The concentration of biomass in R2 was lower compared to R1, probably due to the poor settling performances of granules due to their loose structure and low density, and consequently washed out of the biomass.

Table 4: overall treatment performances expressed as % of removal, calculated from the measured effluent concentrations and the theoretical influent values. Average and standard deviation (SD) were calculated from 10 measurements under one month of operation prior to batch experiments.

	R1	**R2**
Biomass concentration, gTSS L^{-1}	12.6 ± 3.7	8.6 ± 3.2
COD %	96.6 ± 1.8	98.3 ± 0.4
NH_4^+-N %	84.8 ± 12.5	47.6 ± 14.7
N_{tot} %	75.6 ± 12.7	46.2 ± 24.3
PO_4^--P %	50.0 ± 21.9	15.7 ± 28.0

The lower nutrient removal performances in R2 could be attributed to the presence of fungal granules. It is also known that several species of fungi are able to nitrify and denitrify (Field et al., 1993), even at higher rates compared to bacteria (Guest and Smith, 2002). Fungi has also been found to effectively remove phosphorus from dairy manure wastewater (He et al., 2019). It is unclear whether the wide variations of pH in R2 inhibited the fungal capability of nutrient removal. Molecular tools should be employed to analyze the fungal community and identify the species present in the reactor.

The DO provided during the aeration phase is an important operational parameter affecting the performances of the aerobic granular system. The DO level was monitored over a series of cycles and revealed oversaturating conditions during the reaction phase. Oversaturation of DO leads to deep oxygen penetration in the granule, stopping simultaneous denitrification because of inhibition with oxygen (Lochmatter et al., 2013; Meyer et al., 2005). The high oxygen level in the aeration phase also affected the consecutive feeding stage. Low redox conditions during feeding are crucial for biological phosphorus removal to occur and denitrification of the leftover NO_3^- from the previous cycle.

The morphological features of the cultivated granules were analyzed with a light microscope (Figure 10). The granules in R1 showed a dense structure, while in R2 a loose and filamentous structure dominated. Higher magnification in the fungal granule surface revealed large pores of a few hundred µm in size, which allow for efficient substrate and oxygen transport into the central part of the granules. Fungi can be easily distinguished under the microscope at low magnification by their robust branched filaments, with a clearly visible septa that separates cells with a diameter larger than > 2.5 µm.

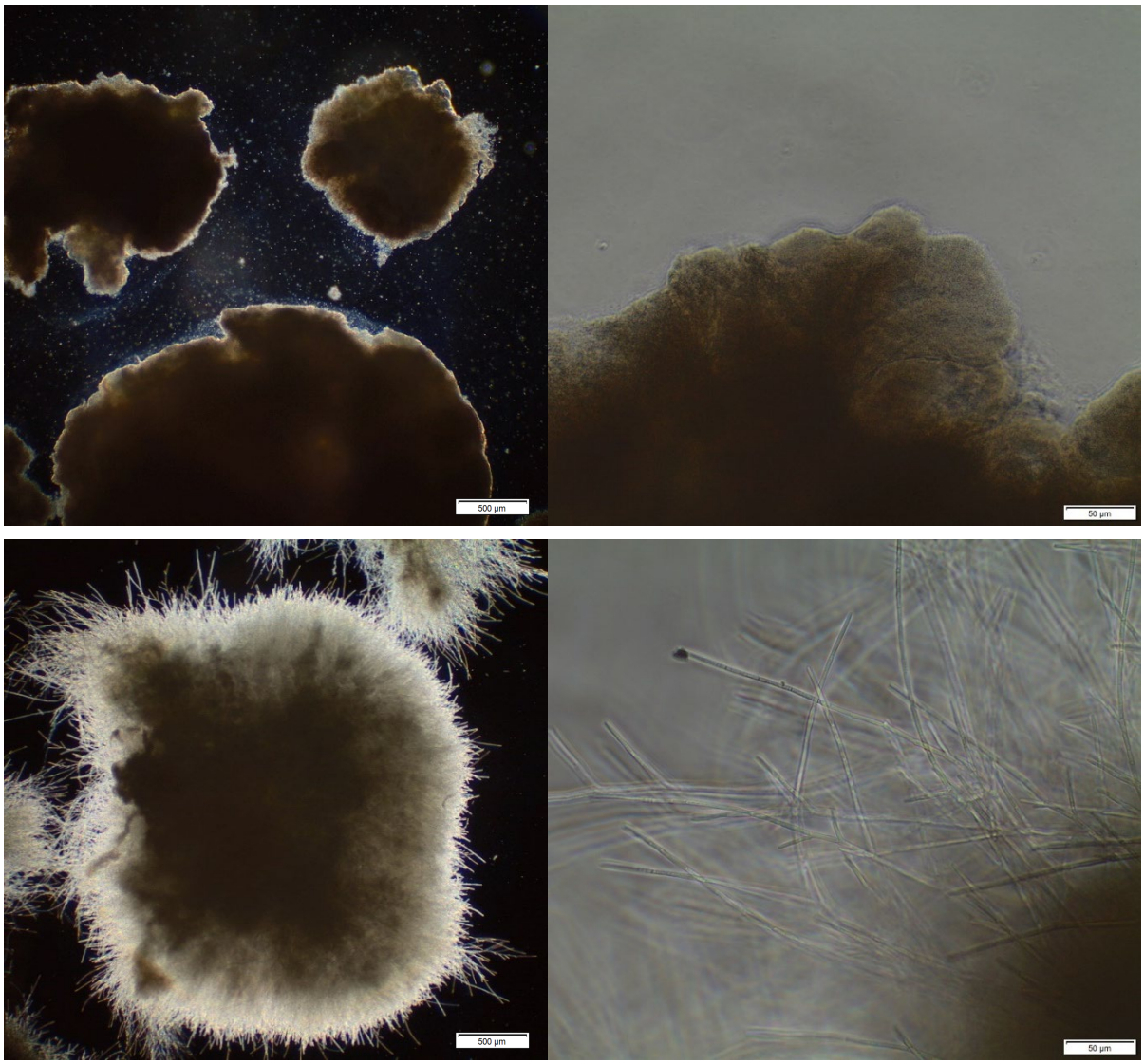

Figure 10: Light microscopy images of the granules cultivated in R1 (first row) and R2 (second row). The fungal granule surface was characterized by abundant branched assembly of filaments (hyphae) forming an entangled mycelium. The images on the left are taken at 2 x magnification (scale bar 500 µm). Right side images are taken at 20 x magnification (scale bar 50 µm).

4.1.2. **AGS reactor for long term exposure to OMPs**

A new lab scale SBR was assembled to obtain more controlled conditions in terms of pH, temperature and DO levels. The first months of operation of the new reactor consisted of testing, calibrating and adjusting the operation of the reactor components (sensors, gas pump, MFCs) to reach the desired process conditions. During this time, the removal performances of the reactor were monitored. OMP were dosed continuously, but the investigation of their concentrations in the effluent could not be performed due to lab closure because of the COVID-19 pandemic. Several disturbances due to the failure of the controlling system occurred. During day 10, 16 and 55 the nutrient pump (C, N) did not deliver the media during the night. Moreover, the increased pressure in the gas recirculating system, due to clogging of the metal sparger, caused the gas pump to break. After this event, the system was running at oversaturating DO conditions during the aeration phase for 20 days (day 29-49).

The evolution of the biomass concentration is described in Figure 11. The settling time was reduced gradually to avoid the wash out of slow growing organisms. The concentration of TSS in the reactor decreased during the first month of operation, to finally increase up to 5.6 gTSS L^{-1} during the second month. A drop of biomass concentration was observed after decreasing the settling time from 10 to 5 min in the third month of operation (after day 64). The appearance of filamentous fungi in the system could be the reason for the lower biomass concentration in the reactor (Figure 13).

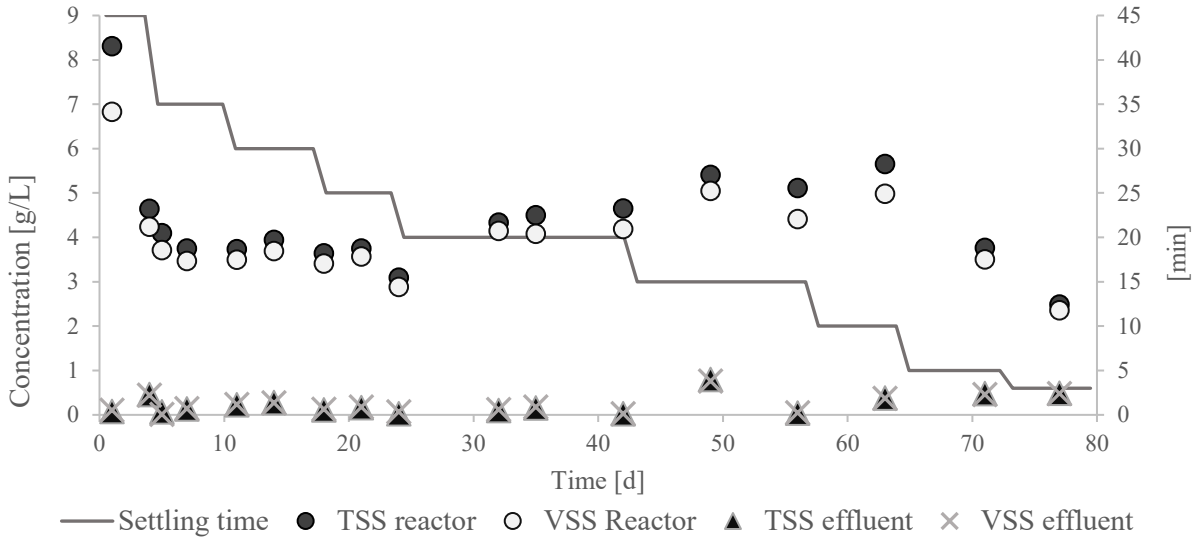

Figure 11: settling time variation, biomass concentration in the reactor and effluent suspended solids expressed as g L^{-1} of TSS and VSS.

The removal efficiencies of the system are described in Figure 12. Clearly, the removal of carbon remained stable for most of the experiment, with an average removal efficiency of 93 ± 6 %. Despite the effluent concentrations of NH_4^+-N varied, nitrification took place in the reactor. A drop in NH_4^+ oxidation was observed during days 24, 42, 53 and 71, which caused the unstable TN removal. NO_2^--N was never detected in the effluent and the NO_3^--N concentrations were always below 3 mg L^{-1}, confirming an efficient denitrification in the

system. The average TN removal efficiency during the run was 80 ± 25 %. The removal efficiency of TP was overall high, except for some occasions in which the performance dropped. The first week of operation phosphorus uptake was not stable, probably due to the biomass adaptation to the new conditions. In days 18 and 24, around 31 % TP was removed. Poor performances were observed from day 56, but increased over the following weeks, until no PO_4^{3-} -P was detected in the effluent. A possible reason for the low TP removal performances could be the failure of the pump delivering the organic carbon and nitrogen sources. Without organic carbon during the anaerobic phase, no PHAs were stored by PAOs. PHAs are a necessary energy source in the aerobic phase for PO_4^{3-} uptake and poly-P storage. The higher removal performances of the new reactor confirmed the importance of controlling DO and pH level in the lab scale system during the aeration phase. Despite the presence of pharmaceuticals in the synthetic wastewater influent, the overall removal performances in terms of carbon, nitrogen and phosphorus did not seem to be affected.

Sporadic fungal filaments were observed from day 42 and increased the following weeks, until they became predominant from day 71. The reason for the fungi overgrowth in the system could be several. In this run, the pH was controlled around neutrality and minimum values were registered at the end of the anaerobic feeding (pH ~ 6.6). The acidic condition was likely due to the glucose degradation following a glycolytic pathway and transformation into VFAs. Maximum values were occurring during the aerobic phase probably due to CO_2 stripping. It is generally believed that the fungi are able to grow with an extremely low level of nutrients (Li et al., 2010). The failure of the pumps delivering the synthetic wastewater during several cycles created a low nutrient condition that could have caused the filaments to bloom. The presence in the feed of pharmaceuticals could be another reason for the fungal overgrowth. It has been reported that exposure of OMPs causes the destabilization of the granules (Amorim et al., 2016; Kent and Tay, 2019; Kong et al., 2015). Compared to bacterial species, fungi have a better resistance to inhibitory substances (Sankaran et al., 2010).

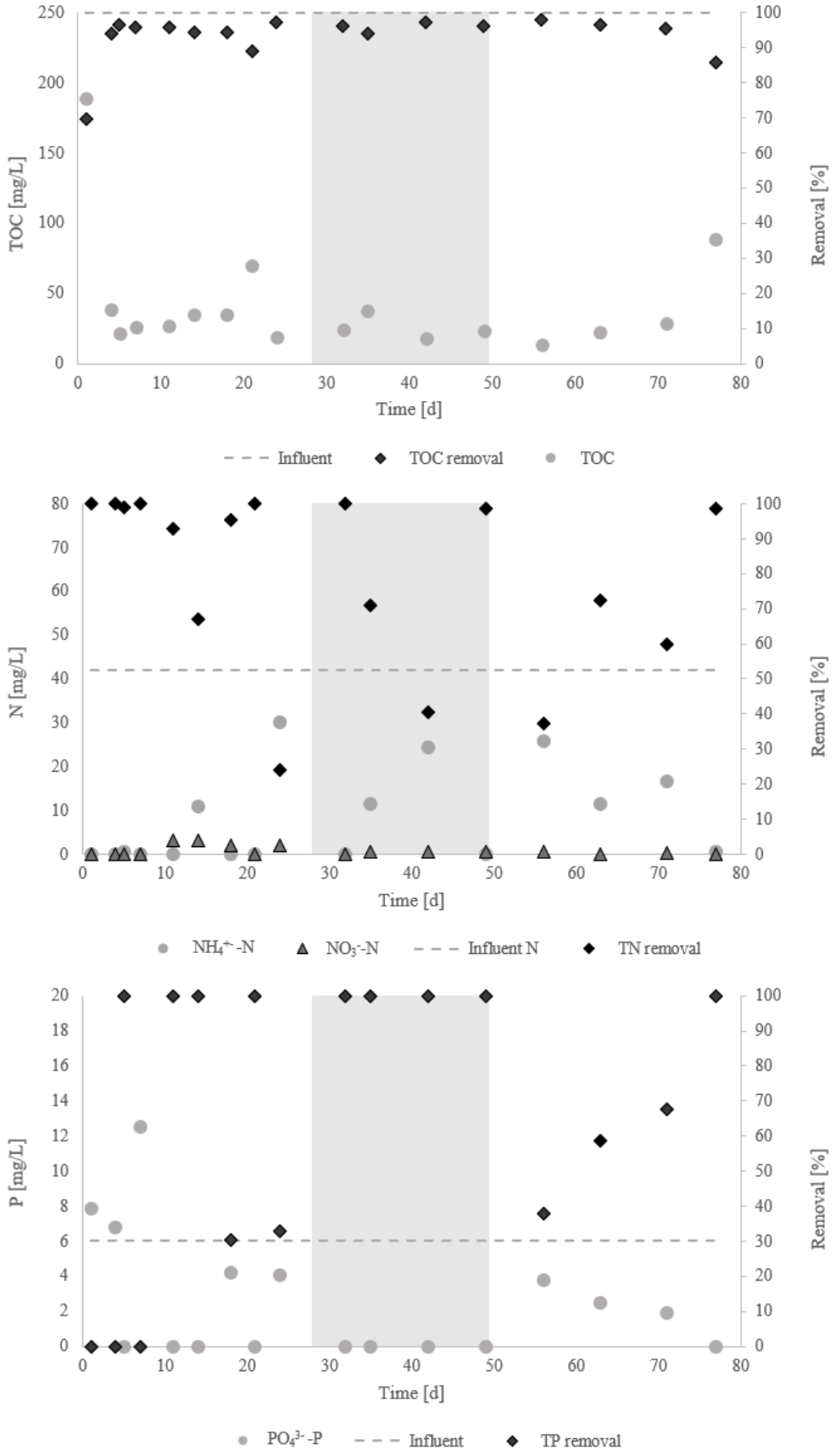

Figure 12: theoretical influent concentrations, measured effluent concentrations and removal performances of the reactor of TOC, N and P. The darker area refers to the period of oversaturated conditions in the aeration phase.

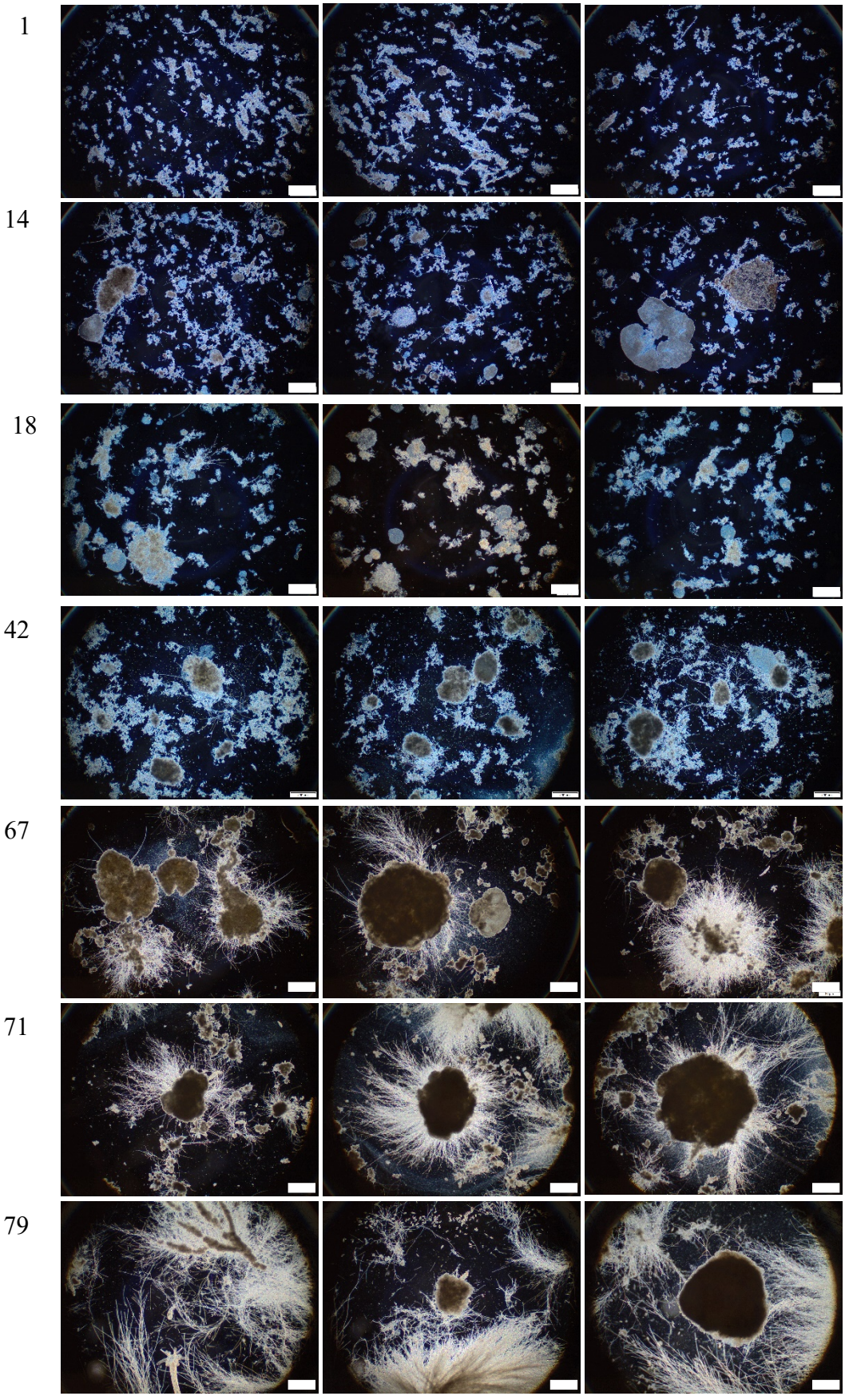

Figure 13: Light microscopy images of the biomass evolution in the reactor observed at 2 x magnification. The scale bars represent 500 µm.

4.2. Sorption test and results

The two biomass conformations used in the sorption test appeared significantly different. Granules dominated by fungi, exhibited a more porous structure and larger surface area compared to the dense bacterial granules (Paper I, Figure 1). Because of the different structure and dominating microorganisms, the two biomass conformations were expected to exhibit different cell hydrophobicity. Wider variation was observed within granules of different diameter dominated by bacteria compared to fungal granules (Paper I, Figure 2). All the granules showed higher affinity for the polar solvents, which revealed the presence of electron donor and electron acceptor functional groups present at the cell surface. The bacterial granules showed lower hydrophobicity with larger size, likely due to the lower fraction of active biomass and higher fraction of inert materials.

Nine pharmaceuticals among the 22 compounds tested, namely CTL, CLY, ERY, E2, EE2, KTC, LEV, LOS and SER, showed removal > 20% in at least one biological sample (Figure 14). Those compounds are mostly positive and hydrophilic or moderately hydrophobic, or neutral or negative and lipophilic. However, removal was also observed in the control batch test and for some compounds, namely CLA, ERY, LOS and LEV, the concentrations measured at the end of the blank test were even lower than the values observed in the liquid phase of the biological inhibited samples. The low analyte recoveries observed in the blank test for ERY, E2, EE2, CLY, KTC, LOS, LEV and SER, likely resulted from various processes, including: (i) volatilization, (ii) sorption to the glassware, (iii) sorption to the filter membrane, (iv) abiotic transformation during the experiment, (v) photodegradation, (vi) abiotic transformation during freezing/sample storage.

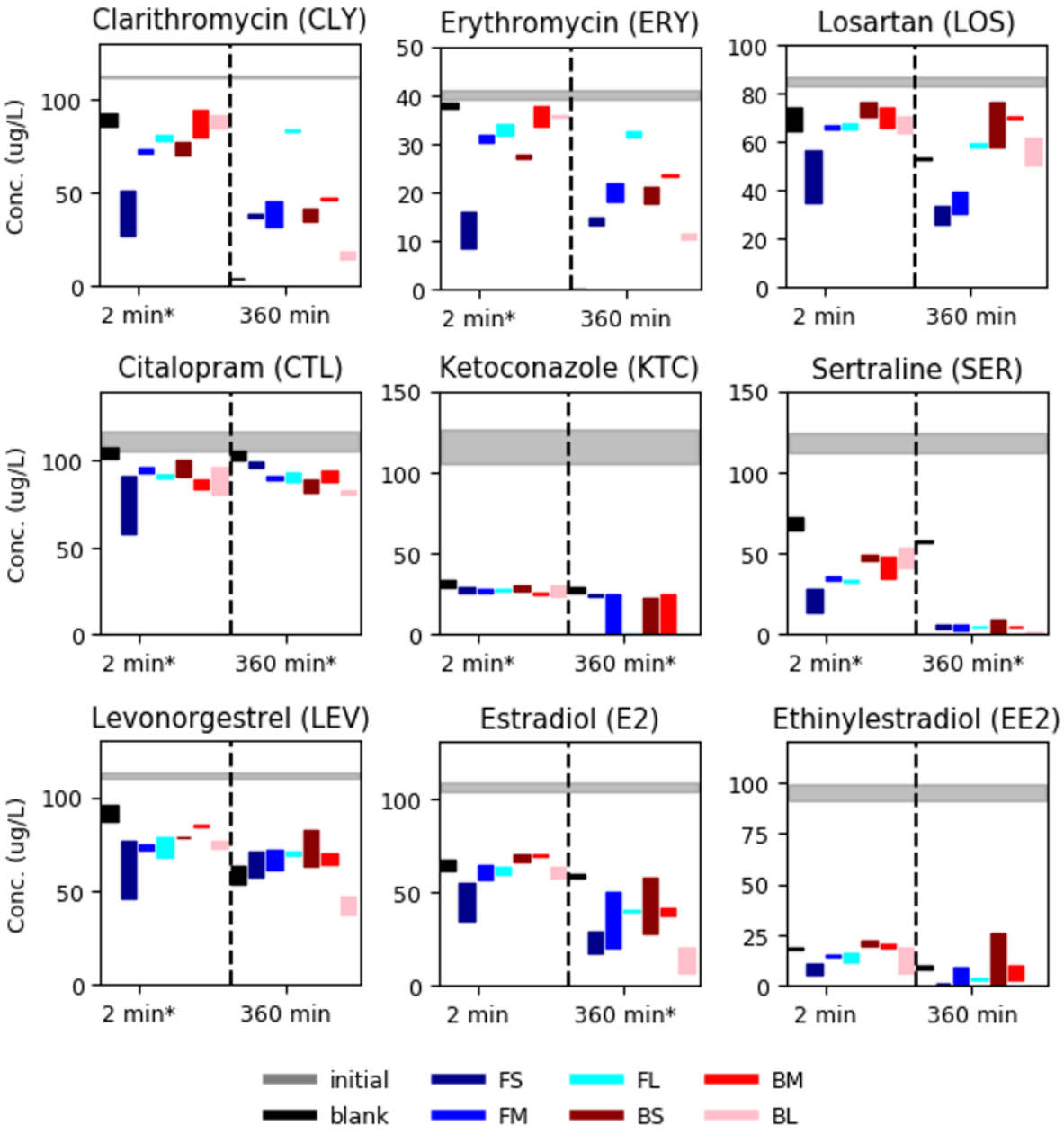

Figure 14: Concentration of the OMPs in the liquid phase during the sorption tests. The bars show the maximum and minimum concentrations from the duplicate samples. An asterisk (*) next to the sorption time on the x-axis indicates that the concentration with biomass is significantly lower than then the average concentration of the blank ($p<0.05$, one sample t-test, $n=12$, all biomass samples combined).

4.3. Full scale study results

All measured compounds were detected in the primary clarified wastewater at average concentrations ranging from 1 ng/L to 43 µg/L, which are in accordance with the concentrations detected and compiled by previous studies in Swedish wastewater (Falås et al., 2012; Paxéus, 2004). The removal efficiencies varied greatly among the compounds (Figure 15), The substances with the highest incoming concentrations were readily removed from the water phase, with an average removal between 80% and 100%. High removal (> 70%) was observed for clarithromycin, sulfamethoxazole, BPA, methotrexate, ciprofloxacin and ketoconazole. Venlafaxine, citalopram, sertraline and estrone showed significant removal (40-70%). The high removal of ciprofloxacin, ketoconazole, venlafaxine and citalopram can be explained by adsorption to sludge (Svahn et al., 2019b). Partial or no removal was obtained for the most persistent OMPs. The activated sludge reactor, being the first reactor in line, contributed to most of the removal from the water phase for the easily degradable compounds. Additional removal of the degradable compounds was observed in the trickling filter and in the MBBR units. Negative removal is reported for compounds having higher concentrations in the effluent compared to the influent. Negative removal might be explained by: (i) desorption processes; (ii) transformation of pharmaceutical conjugates; (iii) inaccuracies of the sampling method; (iv) heavy matrix effects suppressing the MS/MS signal.

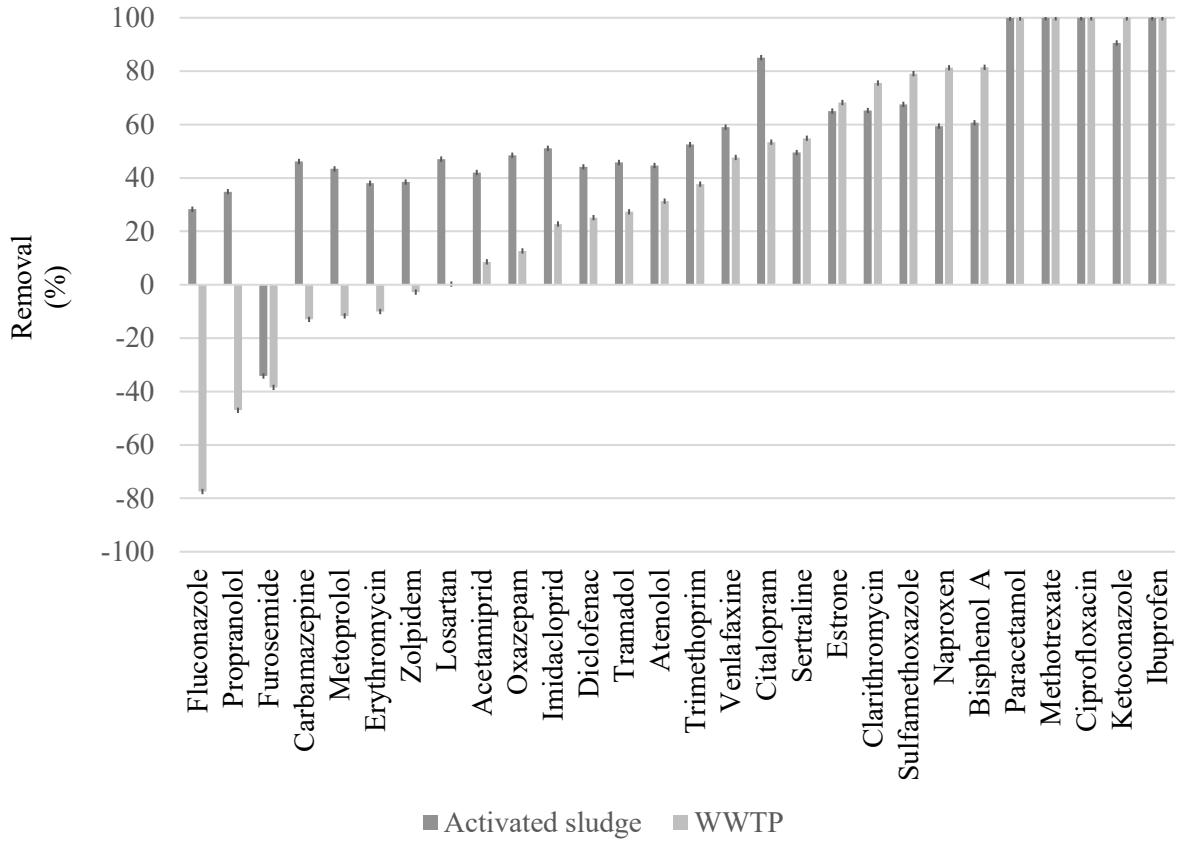

Figure 15: Comparison of average removal efficiencies of the Activated Sludge (AS) unit and of the entire WWTP. The bars indicate standard deviation.

5. Conclusions

- Two parallel reactors inoculated with the same seed sludge and operated at the same nutrient load, showed different removal performances. The reason could be the appearance in one reactor of filamentous microorganisms that under the microscope were recognized as fungi. The acidic pH is likely the cause of fungi overgrowth, therefore keeping the suspension alkaline may be an effective strategy to avoid the dominance in the reactor of these filamentous microorganisms.
- The granules appeared significantly different in terms of morphology. While bacteria dominated granules exhibited smooth surfaces and dense bodies, fungal granules exhibited fluffy and porous structures.
- The results of the hydrophobicity test revealed that bacterial and fungal granules had higher affinity for the polar solvents compared to the nonpolar, suggesting the presence of electron donor and electron acceptor functional groups at the cell surface. The poor affinity to the nonpolar solvents, revealed that the bigger bacterial granules, BM and BL, had weak hydrophobic characteristics compared to the other fractions, probably due to their dense structure and higher inert content.
- Among the 22 investigated OMPs exposed to inhibited granules, nine pharmaceuticals showed removal > 20% during the test in at least one biological sample. When comparing the biosorption capacities of different granules, no correlation was observed with the granule size or biomass characteristics.
- Abiotic transformations of trace organic contaminants, other than biosorption, was observed. To distinguish the removal due to biosorption from other abiotic removal mechanisms, a different method to analyze the sorbed OMPs should be used. Extraction of the sorbed OMPs from the solid matrix and consequent analyses should reveal the true amount sorbed. This method would also avoid the inhibition of the biomass, a step that might change the physico-chemical characteristic of the sludge.
- The new lab scale AGS reactor with controlled DO and pH level allowed for improved performances of nutrient removal in terms of biological phosphorus removal and total nitrogen removal.
- Filamentous fungi appeared in the system and several reasons might be the cause of the fungal bloom. The slightly acidic pH at the end of the anaerobic feeding, the constant exposure to OMPs and the low nutrient conditions (when the carbon and nitrogen pump did not deliver the media) could have provided advantages to the filamentous fungi over the bacterial community. The performances in terms of COD and nutrient removal did not change.
- At the full scale WWTP, the activated sludge reactor contributed to most of the removal of OMPs from the water phase for the easily degradable compounds. The most persistent compounds remained stable through all biological treatment steps.
- The limited removal efficiency provided by the carrier biofilm is partly due to already low influent concentrations, due to efficient removal in the activated sludge system but may also be attributed to the short hydraulic times applied in the MBBRs. This suggests that the optimization of biological treatment strategies towards OMP removal would be restricted by the HRT applied in the units.

6. Implications and further research

Research about OMP removal in the biofilm system is necessary to develop strategies to optimize the elimination in the biological system, to exploit the advantages of biofilm characteristics and to limit the use of resource-intensive physico-chemical treatment technologies.

This research aims at understanding the mechanisms behind the removal of OMPs in biological reactors and focused in a first phase on the sorption potential of different conformations of granular sludge. The next steps in this research project is to investigate the biotransformation potential of AGS systems, with focus on the role of the microbial communities and redox conditions.

To assess how the microbial community composition influences the degree of degradation, the relationship between operational conditions and microbial community and microbial degradation mechanisms will be investigated in long term experiments in well controlled AGS reactors.

One study is aimed to investigate the removal performances under different redox conditions in the aerobic phase. Two oxygen saturation levels will be assessed to study whether an increase of the aeration is beneficial for the removal of OMPs. As supported by many authors, aerobic conditions and the presence of a diversified aerobic heterotrophic microbial community should be favored to increase micropollutant transformation (Falås et al., 2012; Gusmaroli et al., 2020; Margot et al., 2016). With the possibility of controlling the temperature in the reactors, a low temperature study (8° -12° C) will be included to assess the limits of the biological processes at winter conditions, typical for the Nordic countries. Due to the slower metabolic reactions and inhibition of nitrification at low temperature, the removal rates of OMPs is expected to decrease (Kruglova et al., 2016).

Some OMPs are resistant to AS treatment. It is therefore of interest to study whether the elimination can be enhanced in the AGS system. The difference in OMP removal between aerobic granules and activated sludge flocs will be compared in lab scale batch test and at a full-scale treatment plant, where AGS and AS tanks are running in parallel.

The fungal bloom in the reactor gave the opportunity to study OMP removal by the fungal community. Many studies have described the potential of fungi, particularly the white-rot fungus species, to degrade pharmaceuticals from wastewater efficiently (Badia-Fabregat et al., 2017; Hofmann and Schlosser, 2016; Olicón-Hernández et al., 2017). Thanks to their non-specific, extracellular oxidative enzymes, which in nature is involved in lignin degradation, fungi have been observed to facilitate the biodegradation of recalcitrant compounds (Harms et al., 2011). Investigations on the diversity and function of fungi in aerobic granules could lead to possible biotechnological applications.

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