

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Removal of Micropollutants from Wastewater in Aerobic Granular
Sludge and Activated Sludge Systems**

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Cover:

Left: Light microscopy image of aerobic granular sludge dominated by fungal mycelia

Middle: SEM image of the protozoa on the surface of aerobic granular sludge

Right: SIMS image with increased color saturation of different pharmaceuticals in the biological matrix of aerobic granular sludge

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ABSTRACT

The presence of organic substances in the aquatic environment, such as pharmaceutically active compounds, antibiotics, and personal care products, has become a worldwide issue of increasing environmental concern. As they are present at nano- to microgram per liter concentrations, they are defined as organic micropollutants (OMPs). Understanding the removal of micropollutants mediated by biological processes in wastewater treatment plants is the key to developing and deploying strategies to efficiently reduce environmental exposure to such contaminants. The biomass configurations (suspended growth systems or biofilms) can affect the removal of OMPs, and the underpinning mechanisms need to be substantiated. Aerobic granular sludge (AGS) is a form of free-floating biofilm technology for the simultaneous removal of organic carbon, nitrogen, and phosphorus in a single process step. The features of AGS make this technology very attractive for the removal of OMPs, but an in-depth understanding of the fate of OMPs in such systems under different operational conditions is still required.

The present work investigated the removal mechanisms of OMPs in biological treatment processes with a focus on AGS. Removal performances were evaluated by measuring the presence of OMPs in the water phase at both full-scale treatment plants and laboratory-scale reactors. The kinetics of transformation and sorption behavior were assessed in batch experiments with different biomass types. The microbial communities and antimicrobial resistance genes of the activated sludge and granular sludge systems were compared. The spatial distributions of a few pharmaceuticals inside the biological matrix of AGS were imaged and analyzed together with the endogenous biofilm molecules by secondary ion mass spectrometry.

A higher transformation capacity for most of the investigated OMPs was observed for the activated sludge compared to the granular sludge system, both at the full-scale treatment plant and in the batch experiments. Despite the differences in microbial composition and diversity, the two systems shared similar antimicrobial resistance gene profiles. Micropollutant exposure to the biomass or mass transfer limitations in the dense matrix of AGS likely played an important role and could explain the observed differences in OMP removal. Oxidic conditions seemed to support the microbial transformation of several micropollutants with a faster and/or comparable rate compared to anoxic conditions. Sorption of OMPs to the biomass was observed to be an important removal mechanism for a few compounds. Partitioning of the pharmaceuticals to AGS occurred quickly and increased over time for most pharmaceuticals, suggesting that the compounds can penetrate the deeper biofilm matrix. This observation was also confirmed by the chemical analysis of the biofilm matrix of AGS. The spatial distributions of the pharmaceuticals inside the biological matrix of AGS revealed that the interactions between the OMPs and the biomass happen at specific receptor sites distributed across the biofilm.

Keywords: Organic Micropollutants; Pharmaceuticals; Aerobic Granular Sludge; Biofilm; Transformation; Sorption; Wastewater; Microbial Community.

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I. **Burzio C.**, Nivert E., Mattsson A., Svahn O., Persson F., Modin O., Wilén B.M (2021). Removal of organic micropollutants in the biological units of a Swedish wastewater treatment plant. *IOP Conf. Ser.: Mater. Sci. Eng.* **1209** 012016.
- II. **Burzio C.**, Ekholm J., Modin O., Falås P., Svahn O., Persson F., van Erp T., Gustavsson D., Wilén B.M. (2022). Removal of organic micropollutants from municipal wastewater by aerobic granular sludge and conventional activated sludge. *Journal of Hazardous Materials*, DOI: 10.1016/j.jhazmat.2022.129528.
- III. **Burzio C.**, Mohammadi A. S., Smith S., Abadikhah M., Svahn O., Modin O., Persson F., Wilén B.M. Pharmaceutical sorption to aerobic granular sludge and air-induced foam, *Submitted*.
- IV. **Burzio C.**, Mohammadi A. S., Malmberg P., Modin O., Persson F., Wilén B.M. Chemical imaging of pharmaceuticals in biofilms for wastewater treatment using Secondary Ion Mass Spectrometry, *Under Review*.
- V. **Burzio C.**, Ekholm J., Tang K., Mohammadi A. S., Abadikhah M., Modin O., Persson F., Wilén B.M. Dissolved oxygen concentration impacts nutrient and pharmaceutical removal by aerobic granular sludge, *Manuscript*.

The author of this thesis made the following contributions:

Paper I: Conceptualization of the research goals and aims; Result analysis and visualization; Writing the original draft; Editing and revising the draft after co-author and external reviewer feedback.

Paper II: Conceptualization of the research goals and aims; Sample collection at the treatment plant; Planning and performing the experiments; Result analysis and visualization; Formal Analysis; Writing the original draft; Editing and revising the draft after co-author and external reviewer feedback.

Paper III: Conceptualization of the research goals and aims; Reactor maintenance; Sample collection; Planning and performing the experiments; Result analysis and visualization; Formal Analysis; Writing the original draft, editing, and revising.

Paper IV: Conceptualization of the research goals and aims; Development of methodology; Planning and performing the experiments; Result analysis and visualization; Formal Analysis; Writing the original draft; Editing and revising the draft after co-author and external reviewer feedback.

Paper V: Assembling the reactor; Designing the operation of the reactor; Conceptualization of the research goals and aims; Planning and performing the experiments; Reactor maintenance and sampling; Investigation, Formal Analysis, Writing the original draft, editing, and revising.

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Wilén B.M., **Burzio C.**, Ekholm J., Svahn O., Persson F., Modin O., de Blois M., Gustavsson D. (2022). Biologisk rening av organiska mikroföroreningar. Rapport Nr 2022–8. Svenskt Vatten.

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LIST OF ACRONYMS AND ABBREVIATIONS

AGS: aerobic granular sludge
AOB: ammonia-oxidizing bacteria
AMO: ammonia monooxygenase
ARG: antimicrobial resistance gene
CAS: conventional activated sludge
COD: chemical oxygen demand
DO: dissolved oxygen
DOC: dissolved organic carbon
EPS: extracellular polymeric substance
GAO: glycogen-accumulating organism
HRT: hydraulic retention time
MATS: microbial adhesion to solvents
MBBR: moving bed biofilm reactor
MS/MS: tandem mass spectrometry
NOB: nitrite-oxidizing bacteria
OLR: organic loading rate
OMP: organic micropollutant
PAO: polyphosphate accumulating organism
PE: population equivalent
PHAs: polyhydroxyalkanoates
SEM: secondary electron microscopy
SBR: sequencing batch reactor
SRT: solids retention time
SS: suspended solids
SVI: sludge volume index
TN: total nitrogen
TOC: total organic carbon
ToF-SIMS: Time of Flight Secondary Ion Mass Spectrometry
VFA: volatile fatty acid
VSS: volatile suspended solids
WWTP: wastewater treatment plant

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1. Preface

Anthropogenic chemicals such as pharmaceuticals, illicit drugs, personal care products, and pesticides, have been found in the aquatic environment at trace levels. These organic compounds, also called micropollutants because of their low concentrations, are emitted into the natural environment during their manufacturing, use, and disposal. There is evidence that environmental exposure to organic micropollutants (OMPs) has deleterious effects on the health of ecosystems and humans, e.g., by selecting antimicrobial resistant bacteria, feminizing fish, and increasing the susceptibility of fish to predation (Wilkinson et al., 2022).

Conventional wastewater treatment plants (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. Treatment processes such as adsorption onto activated carbon, oxidation by ozone, and membrane systems have been found efficient for the 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 solids retention time, the high 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. Aerobic granular sludge (AGS) is a biofilm technology where the microorganisms grow in dense aggregates with excellent settling, higher biomass retention, and capability of organic matter, nitrogen, and phosphorus removal, characteristics that enable a small footprint and flexible mode of operation. This form of free-floating biofilm harbors a very diverse microbial community which has been found promising for the removal of toxic compounds. The characteristics of AGS, together with the long solid retention time, 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.

As the mechanisms of OMP removal, the efficacy of transformation by biological units, and the role of microbial communities are poorly understood, the predictivity for removing specific trace organic contaminants remains very limited.

1.1. Objectives and thesis outline

The overall goal of this research project was to study the elimination mechanisms of OMPs from wastewater in the biological treatments at WWTPs. Particular attention was directed to the removal potential of AGS, both at a full-scale treatment plant and in laboratory-scale reactors. Due to the significance of contamination for municipal wastewater, the focus was laid on pharmaceuticals.

The results of this work are summarized in five manuscripts (Papers I-V), in which the following core questions were addressed:

- *What is the contribution of biological treatments to the removal of OMPs in wastewater treatment and does AGS technology present additional benefits compared to the conventional activated sludge process?*

Paper I investigated the removal of OMPs along the biological units of a large Swedish WWTP. In **Paper II**, the removal efficiency of OMPs by AGS and conventional activated sludge was evaluated at another Swedish full-scale WWTP, where the two biological systems are operated in parallel.

- *How do redox conditions and dissolved oxygen in a biological reactor impact the removal of pharmaceuticals?*

In **Paper II**, the transformation rates of selected OMPs were compared between AGS and conventional activated sludge in separate batch tests under oxic and anoxic conditions. **Paper V** investigated the impact of dissolved oxygen on the removal efficiencies of nutrients and pharmaceuticals by AGS in a lab-scale reactor.

- *What is the role of sorption and where in the biofilm do OMPs localize?*

In **Paper III**, the biosorption capacity of aerobic granular sludge was quantified in two types of aerobic granular sludge, one dominated by fungi, and one dominated by bacteria, at different granule sizes. **Paper IV** presented a powerful new strategy for chemical imaging of biofilm for wastewater treatment that was used to identify and localize pharmaceuticals in the complex biological matrix of AGS.

This work is structured in the following chapters: Chapter 2 introduces biological wastewater treatment, the AGS technology, and an overview of the fate of OMPs in biological treatment processes. Chapter 3 briefly describes the materials, reactors, and methods used to investigate the aims of this thesis. Detailed descriptions of the materials and analyses are presented in the original papers. Chapter 4 is devoted to answering the core questions and discussing the findings of this work. The main conclusions and suggestions for future research are provided in Chapters 5 and 6, respectively.

1.2. Scientific approach and limitations

Full-scale WWTP investigations were performed to assess the concentrations of OMPs in wastewater and estimate their removal in biological processes. Full-scale WWTPs are complex systems that are characterized by site-specific wastewater, fluctuations in conditions, and the presence of many concomitant compounds. To reduce the complexity, laboratory-scale systems can be used to study the mechanisms of OMP removal and develop fundamental understanding. A significant part of this project focused on optimizing a lab-scale AGS reactor design and operation to achieve efficient nutrient removal. To study the nutrient and OMP treatment performances, and bacterial community structure of AGS, a well-controlled laboratory-scale system was necessary for this type of investigation. A new reactor was assembled with the capability to control dissolved oxygen level, pH, and temperature, which are factors that can significantly affect OMP removal performances. The components of the reactor were assembled, calibrated, and adjusted to deliver stable operations. The operational conditions in terms of the design of the different phases, cycle length, settling strategy, and synthetic wastewater were also adjusted to mimic full-scale treatment conditions and promote nitrification, denitrification, and biological phosphorus removal. During the realization of this part, other AGS reactors were used to cultivate granules used in the sorption study. These reactors were not optimized for nutrient removal and the lack of dissolved oxygen and pH control hindered complete phosphorus and nitrogen removal.

This research investigated the fate in biological systems of micropollutants intended as parent compounds. Removal was therefore intended as the absence of the parent compound rather than mineralization of the micropollutant.

The AGS was cultivated in a sequencing batch reactor and incoming wastewater entered the vessel during an anaerobic feeding phase. Even though the transformation of some OMPs could occur also in anaerobic conditions, the kinetics investigations in this project were limited to oxic and anoxic conditions.

2. Background

The liquid waste generated by a human community – wastewater – constitutes the water consumed by 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 throughout 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 to 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, antibiotics, 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^{-1} – $\mu\text{g L}^{-1}$). 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 hotspots for antimicrobial resistance (Berendonk et al., 2015), potentially leading to an environmental and human health risk. Although there are no legal discharge limits for OMPs from WWTPs into the environment in the European Union, some micropollutants (i.e., diclofenac, ciprofloxacin, venlafaxine) have been included in the watch list within the European Water Framework Directive (EC, 2022). The list includes chemicals that might represent a significant risk to or via the aquatic environment, but for which data are still insufficient to support their prioritization and must therefore be monitored in freshwater by Member state countries (Merrington et al., 2021). The regulation of the release of OMPs from WWTPs into the environment is a topic of discussion and the treatment plants might face in the coming future new treatment objectives concerning the emissions of those substances (Rizzo et al., 2019).

2.1. Biological wastewater treatment processes

Municipal WWTPs typically include a preliminary treatment and a primary treatment step, a secondary biological treatment process, and a tertiary treatment in case the quality of the water effluent after the secondary treatment is not at the required discharge limit (Figure 1) (Metcalf & Eddy, 2004). 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 (or settlers). 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 new microbial biomass. The most common biological process is called activated sludge, also known

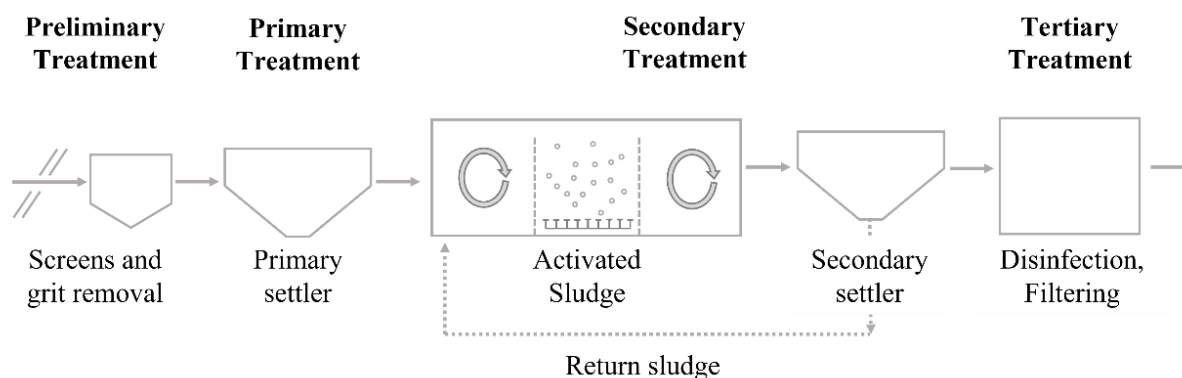


Figure 1: Schematic representation of a conventional wastewater treatment plant.

as conventional activated sludge (CAS), and it involves the production of an activated mass of microorganisms, called biomass, capable of aerobic treatment of wastewater (Metcalf & Eddy, 2004). Separation methods, such as settling, or filtration are then used to separate the biomass and uncouple the hydraulic retention time from the solid retention time. Disinfection and removal of suspended solid is part of a tertiary step.

2.1.1. Main biological processes and microbiology of wastewater treatment

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, i.e., the community of microorganisms found in wastewater, includes Bacteria, Archaea, Fungi, Protozoa, and Metazoa (Figure 2). Protozoa 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). These organisms possess characteristic shapes and are larger than Bacteria (bacterial cell length normally varies from 1 to 5 μm) (Eikelboom, 2000). Figure 2 shows the various types of microorganisms (Fungi, Protozoa, and Metazoa) observed with a light microscope in different biological reactors at full-scale and lab-scale during the investigations performed in this project.

The degradation and mineralization of nutrients and organic matter are executed through a series of enzymatic reactions. Enzymes are intracellular or extracellular proteins that act as biological catalysts. Organic matter degradation is performed by chemoorganoheterotrophic bacteria, which metabolize organic carbon as a 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).

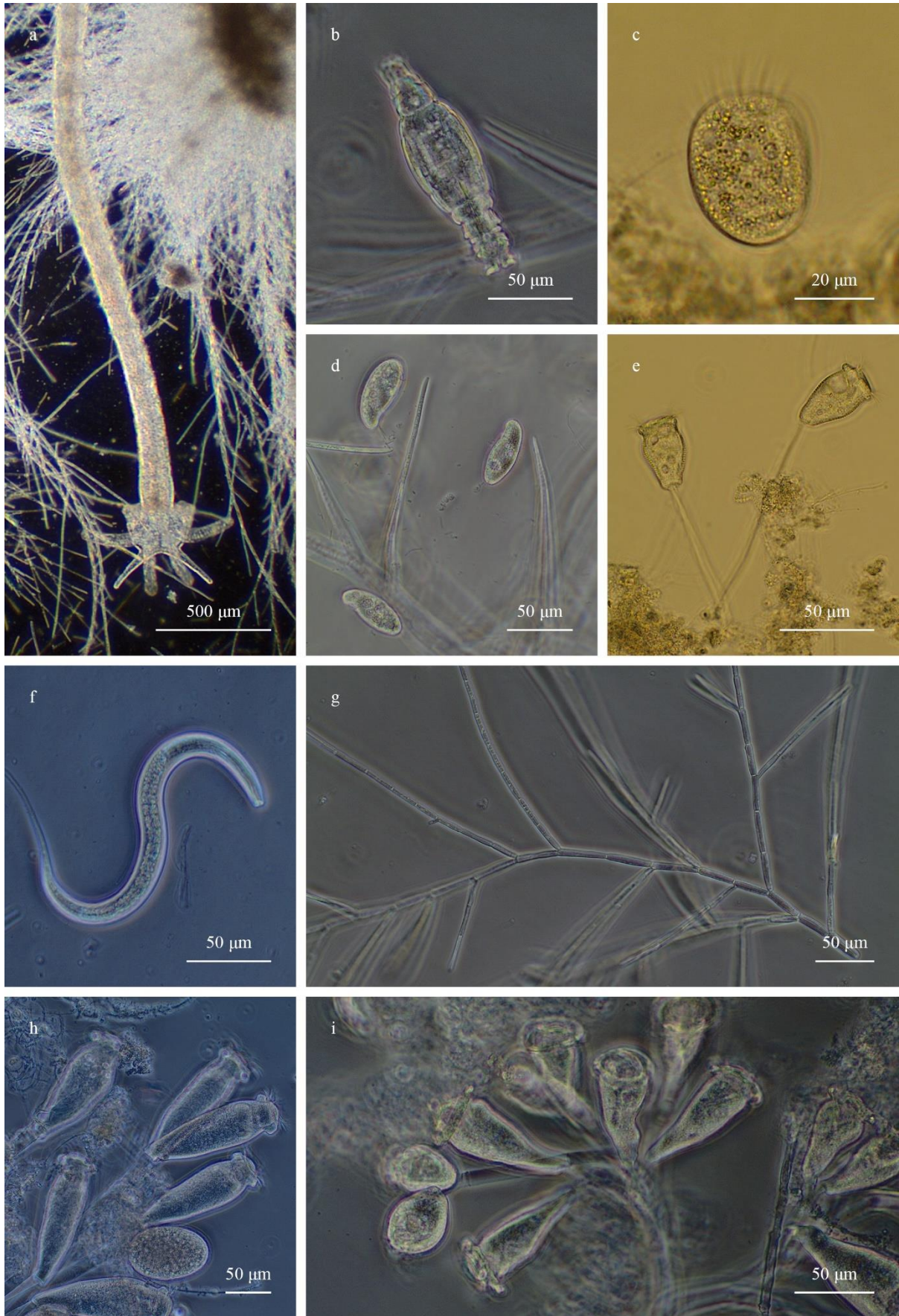


Figure 2: Metazoa (a, b, f), Protozoa (c, d, e, h, k), and Fungi (g) observed in activated sludge and aerobic granular sludge with a light microscope under different magnifications.

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 organisms to nitrate (NO_3^-), through two sequential oxidation processes. In nitrification, aerobic ammonia oxidizing bacteria (AOB) and archaea (AOA) oxidize ammonium into hydroxylamine (NH_2OH) using the enzyme ammonia monooxygenase (AMO). This is followed by NH_2OH oxidation to nitrite NO_2^- , catalyzed by the enzyme hydroxylamine oxidoreductase (HAO). In nitrification, nitrite is oxidized to nitrate in the presence of oxygen by nitrite oxidizing bacteria (NOB) using the nitrite oxidoreductase (NXR) enzyme (van Loosdrecht et al., 2016). Denitrification is the reduction of NO_3^- or NO_2^- (used as an 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) (Rajta et al., 2020). Nitrogen removal can also be performed by anaerobic ammonium oxidation (anammox) bacteria, which anaerobically oxidize ammonium and reduce nitrite producing dinitrogen gas (van Loosdrecht et al., 2016). Complete conversion of ammonia into nitrate can be also performed by a single organism (Comammox) (Gottshall et al., 2021). Nitrogen is removed also through assimilation into the biomass.

The biological removal of phosphorus is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up phosphate intracellularly in the form of polyphosphate (poly-P) granules when exposed to alternating anaerobic and aerobic environments and/or anoxic conditions (Roy et al., 2021). In the anaerobic phase, organic matter is fermented into volatile fatty acids (VFAs). The VFAs are then stored intracellularly as polyhydroxyalkanoates (PHAs) by PAOs (Roy et al., 2021). The energy necessary for this biotransformation is provided by the breakdown of the two intracellularly stored polymers poly-P and glycogen, 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. Unlike the classical PAO organism such as *Ca. Accumulibacter*, *Tetrasphaera* PAOs ferment amino acids and sugars without necessarily cycling PHAs (Close et al., 2021; Nielsen et al., 2019). Finally, PO_4^{3-} is removed from the wastewater by removing the poly-P rich biomass (Metcalf & Eddy, 2004). When exposed to anoxic conditions, nitrogen and phosphorus can be simultaneously removed by denitrifying PAOs (d-PAOs), which use nitrate as the final electron acceptor instead of oxygen (Roy et al., 2021). Nitrate existing in the anaerobic stage could be used as an electron acceptor for the growth of denitrifying heterotrophs that are not PAOs. Their presence reduces the amount of substrate available for PAOs, and hence causes the reduction of phosphorus release.

2.1.2. Biomass configuration

Biological wastewater treatment technologies can normally be categorized into (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 density. Trickling filters 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 using 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 of 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, and predation from some protozoan grazers (Flemming and Wingender, 2010). The distribution and the proportion of the different fractions of polymers determine the physicochemical characteristic of the granule (Seviour et al., 2012). Compared to activated sludge, which commonly comprises small uniform-sized flocs and free-swimming organisms (Figure 3a), the AGS is composed of large granules, from 0.2 to > 4 mm in diameter (Figure 3b, c, and d).

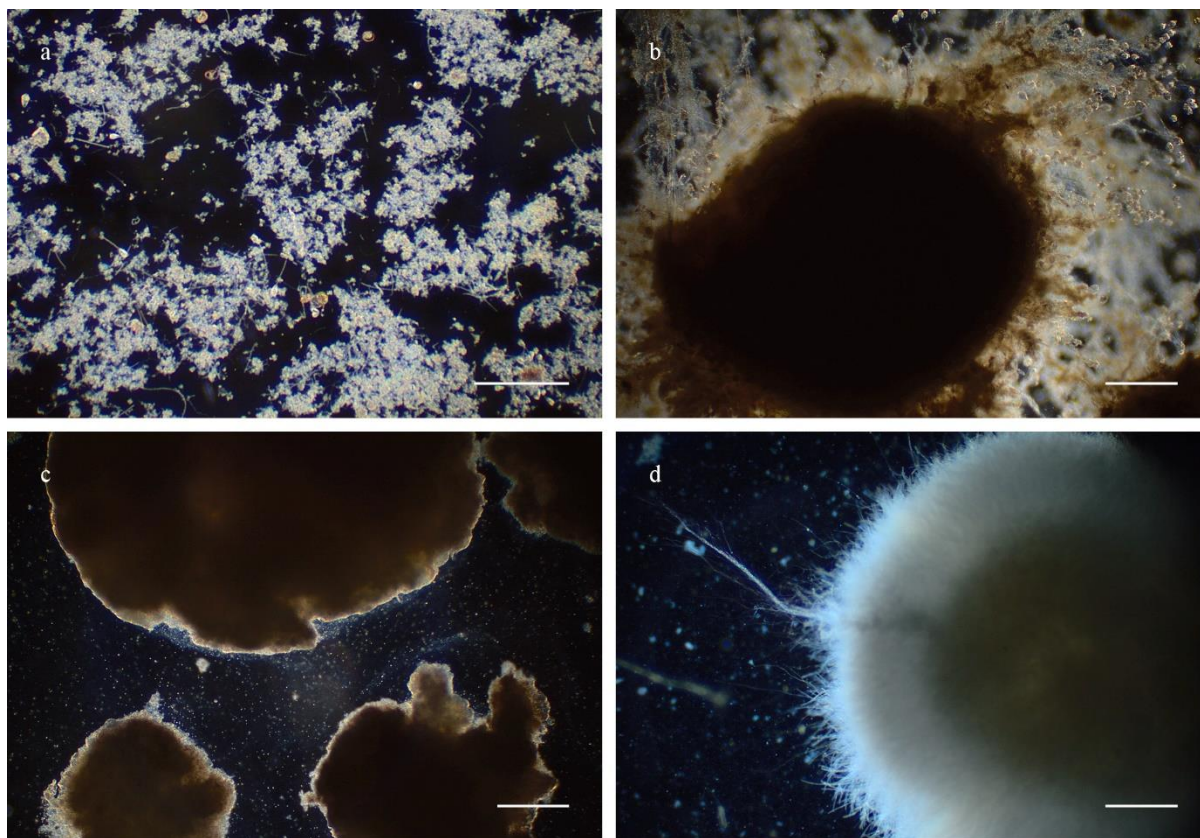


Figure 3: Light microscopy images of the activated sludge (a) and the AGS biological reactors in a full-scale WWTP located in Strömstad (**Paper II**) (b). Granules cultivated in lab-scale reactors (**Paper III**) (c, d) can assume different morphology depending on operating conditions and appear very different from the full-scale ones (b). The images are taken at 2x magnification (scale bar 500 μm).

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 organic matter and nutrients more efficiently (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 up-flow gas rates, large height to diameter ratio of the reactor, and short settling time to wash out slow settling microorganisms.

This biofilm structure incorporates water channels that allow the transport of nutrients and electron acceptors. Because of the size and structure of this biofilm, mass transfer is limited by diffusion, which leads to concentration gradients of electron-donors and acceptors within the granules (Layer et al., 2020). The creation within the granule of gradients of redox conditions and different substrates 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 4).

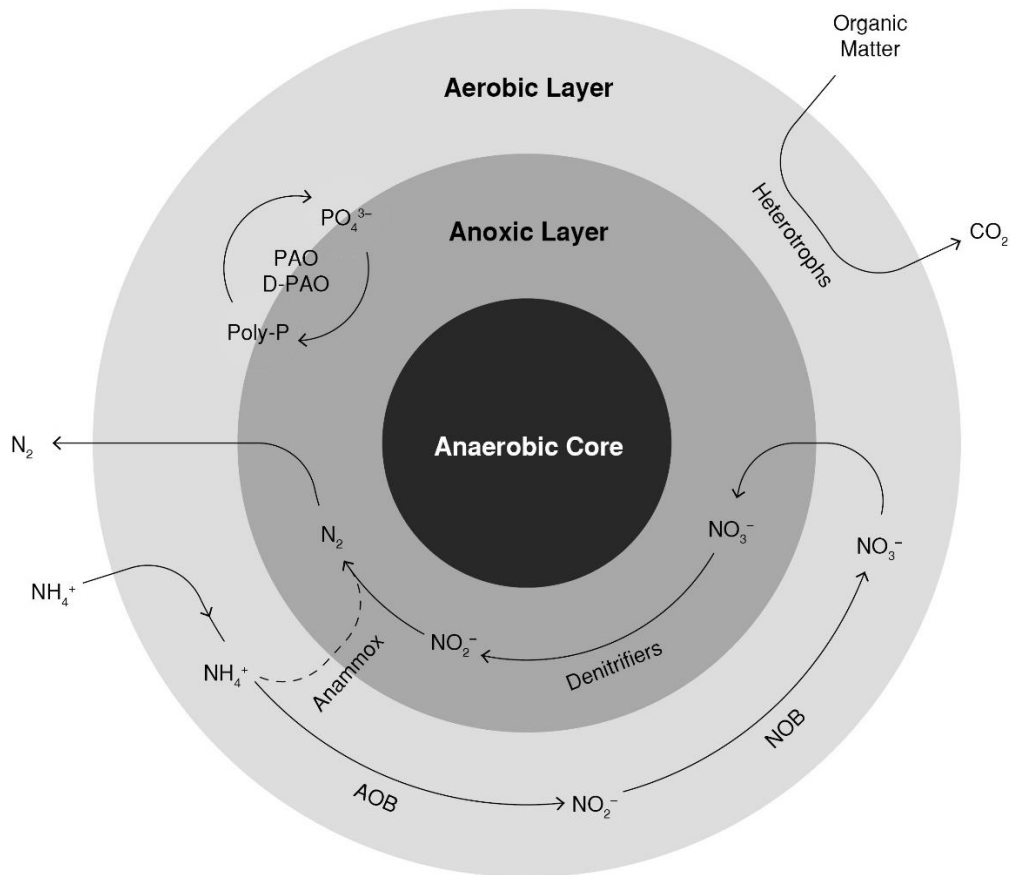


Figure 4: Schematic representation of the structure of an aerobic granule and the biological processes occurring inside.

The advantages of this technology compared to the traditional activated sludge process are several: (i) denser biomass structure with excellent settling capacity; (ii) higher biomass concentration; (iii) capability of simultaneous organic carbon, nitrogen, and phosphate removal (Figure 5); (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 compact reactor with reduced footprint and cost, and also more efficient energy usage due to the lack of mixers and pumping of return sludge and nitrate recirculation (Bengtsson et al., 2018; Pronk et al., 2015). The features of AGS, together with the good biosorption properties and the higher resistance to toxicity, make this technique also 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 for adjusting the length of different phases and redox conditions, can be an advantage when optimizing the system for the removal of trace organic pollutants. More information about the impact of process configurations in the removal of OMPs can be found in paragraph 2.2.3.

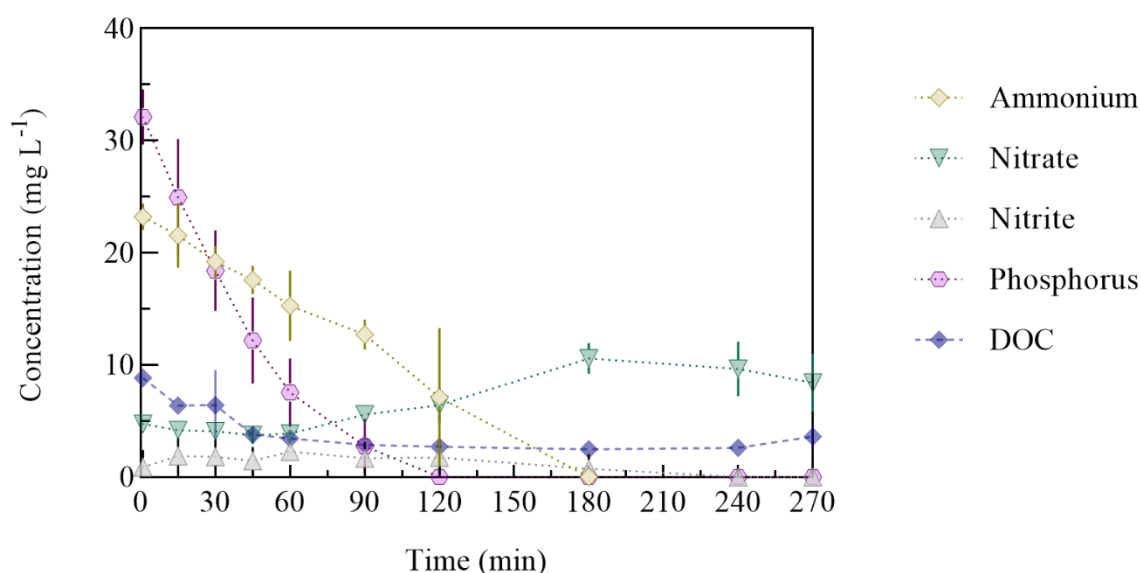


Figure 5: Simultaneous removal of dissolved organic carbon (DOC) and nutrients along the aeration phase (reaction phase) of a lab-scale AGS reactor (**Paper V**). Error bars represent standard deviation from triplicate observations. The low value of DOC at the beginning of the aeration is the consequence of the anaerobic uptake by PAOs and GAOs, resulting in a concurrent release of phosphorus.

2.2. Fate of organic micropollutants in biological treatment processes

A large number of emerging OMPs have been detected in numerous different environments (Figure 6). Several sources contribute to the presence of OMPs in environmental compartments, including (i) human consumption (e.g., pharmaceuticals, personal care products, and drugs of abuse), (ii) manufacturing industry, and (iii) veterinary and agricultural use (e.g., antibiotics and pesticides).

In the aquatic environment, pharmaceutical compounds are one of the most frequently detected classes of OMPs (Rivera-Utrilla et al., 2013). Pharmaceuticals for human consumption can be administered orally, intramuscularly, topically, or intravenously. After the use, the active compounds get excreted and residual concentrations of pharmaceuticals enter the flow of urban wastewater. 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; Verlicchi et al., 2012). Residual concentrations can spread to surface waters, groundwater, or sediments (Figure 6). When sewage sludge is used as soil fertilizer, OMPs can spread onto agricultural land and reach the groundwater (Kümmerer, 2009).

Other common points of entry are hospital and manufacturer wastewaters, and leachates from landfill sites. Veterinary pharmaceuticals directly enter the surface water or through the manure of treated livestock (Le Page et al., 2017).

Due to their vast application, persistence, mobility, and bioaccumulation properties, these chemicals are ubiquitous and are found even in aquatic wildlife as a result of their uptake from the environment (Cervený et al., 2021).

Concerns about antibiotic compounds, antimicrobial resistance genes (ARGs), and antimicrobial resistant bacteria in the environment have also been rising due to the increasing occurrence of pathogens resistant to antibiotics (Berendonk et al., 2015). Because of the high density and diversity of microorganisms exposed to antibiotic residues at sub-inhibitory concentrations, WWTPs are also identified as hotspots for resistance development and ARG dissemination (Rizzo et al., 2013).

When advanced physicochemical treatments such as ozonation or activated carbon adsorption are not implemented, the effectiveness of the biological treatment units determines the performance of the plant in eliminating OMPs. Yet, the mechanisms of removal, the efficacy of the biological units in mineralizing the compounds, and the toxicity of OMPs on 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).

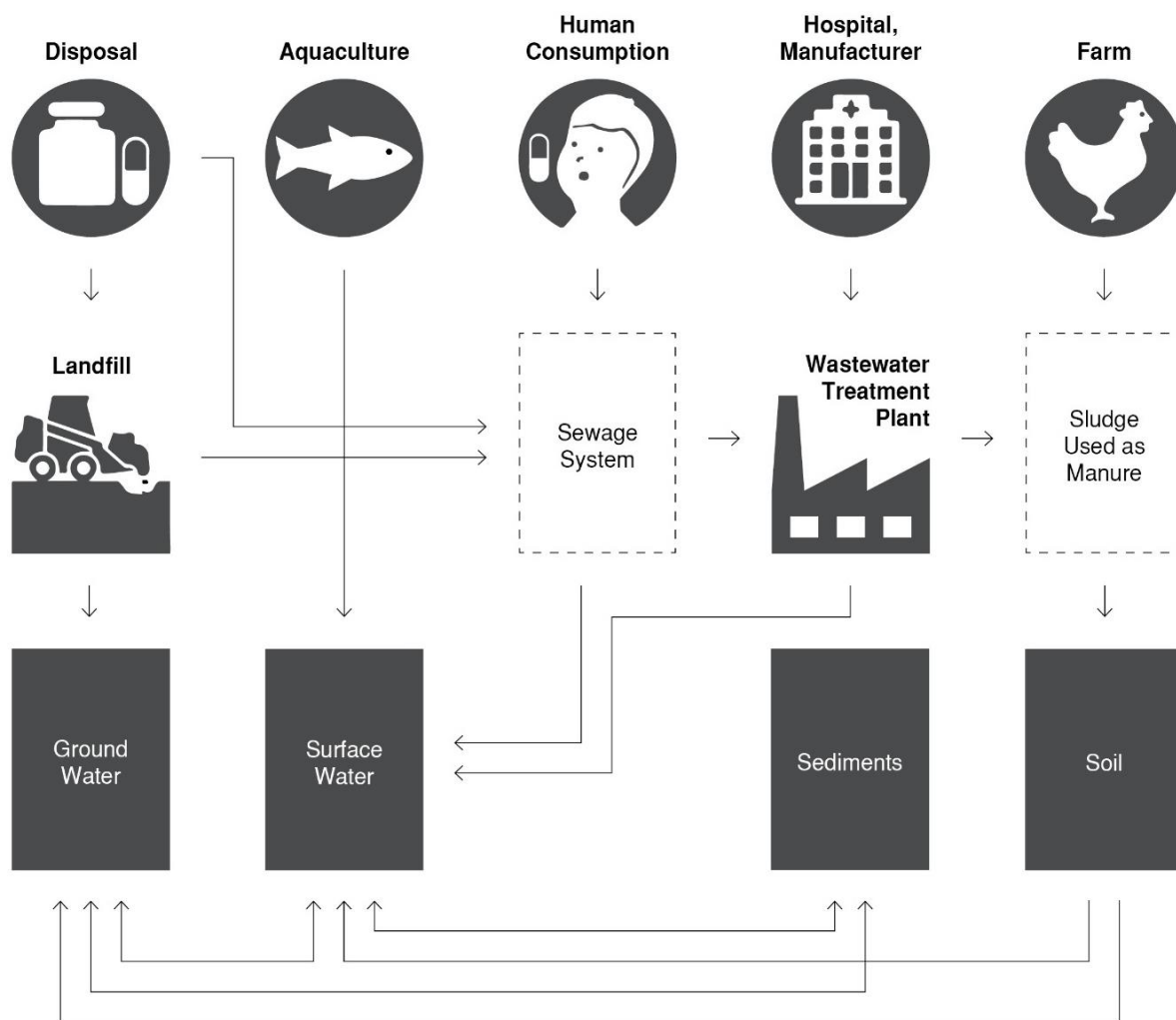


Figure 6: Common points of entry of pharmaceuticals and personal care products and routes into the environment.

2.2.1. Molecular characteristics of micropollutants

Significant variations in the biological 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). The 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, and 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 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.

2.2.2. Removal mechanisms of micropollutants

In biological wastewater treatment processes, the removal of OMPs from the aqueous phase is driven by two main mechanisms: biotransformation and sorption to biomass (Figure 7). Transformation driven by biological processes results in the modification of the chemical structure of the parent compound to metabolites or transformation products, which can be less biodegradable and more toxic than the parent compound (García-Galán et al., 2016).

While biotransformation modifies the compound structure, 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) (Figure 6). 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, volatilization, sorption to colloids, and adsorption to the foam.

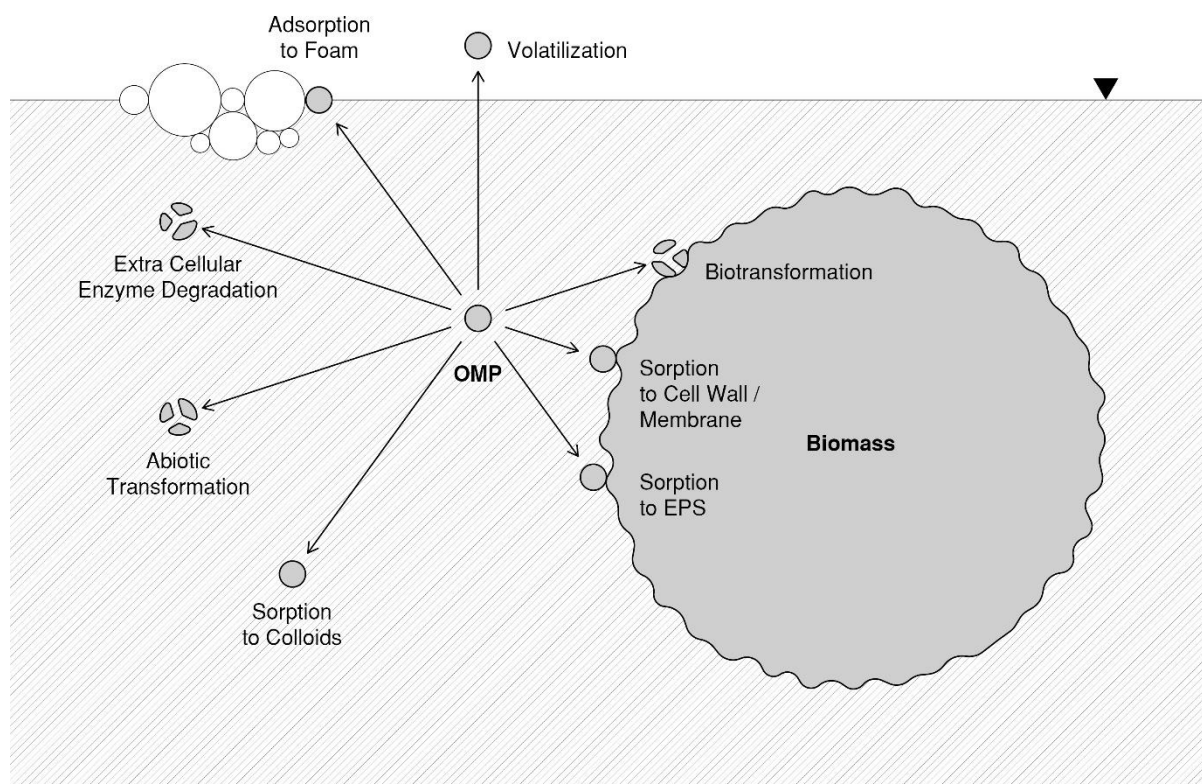


Figure 7: Removal mechanisms of OMPs from the aqueous phase in biological wastewater treatment processes.

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). It is uncertain whether OMPs are removed by direct metabolism, cometabolism, or as secondary substrate in biological systems (Çeçen and Tezel, 2017). OMPs are generally recognized as non-growth substrates as the concentration of OMPs is too low to sustain biomass growth. As a result, cometabolic biodegradation is believed to be the dominating biodegradation process (Nsenga Kumwimba and Meng, 2019), i.e. OMPs 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 that 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). It

is difficult to discriminate the OMP removal by secondary substrate utilization or cometabolism. The removal mechanisms are different and, unlike a secondary substrate, a cometabolic substrate cannot be degraded even at higher concentration as a primary substrate (Çeçen and Tezel, 2017).

Sorption onto biomass

Sorption onto biomass (or biosorption) can be defined as a metabolically passive physicochemical process involved in the binding of ions or molecules from an aqueous solution onto the surface of a sorbent of biological origin. The degree of distribution of the micropollutant molecules in the water phase and 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 8), 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

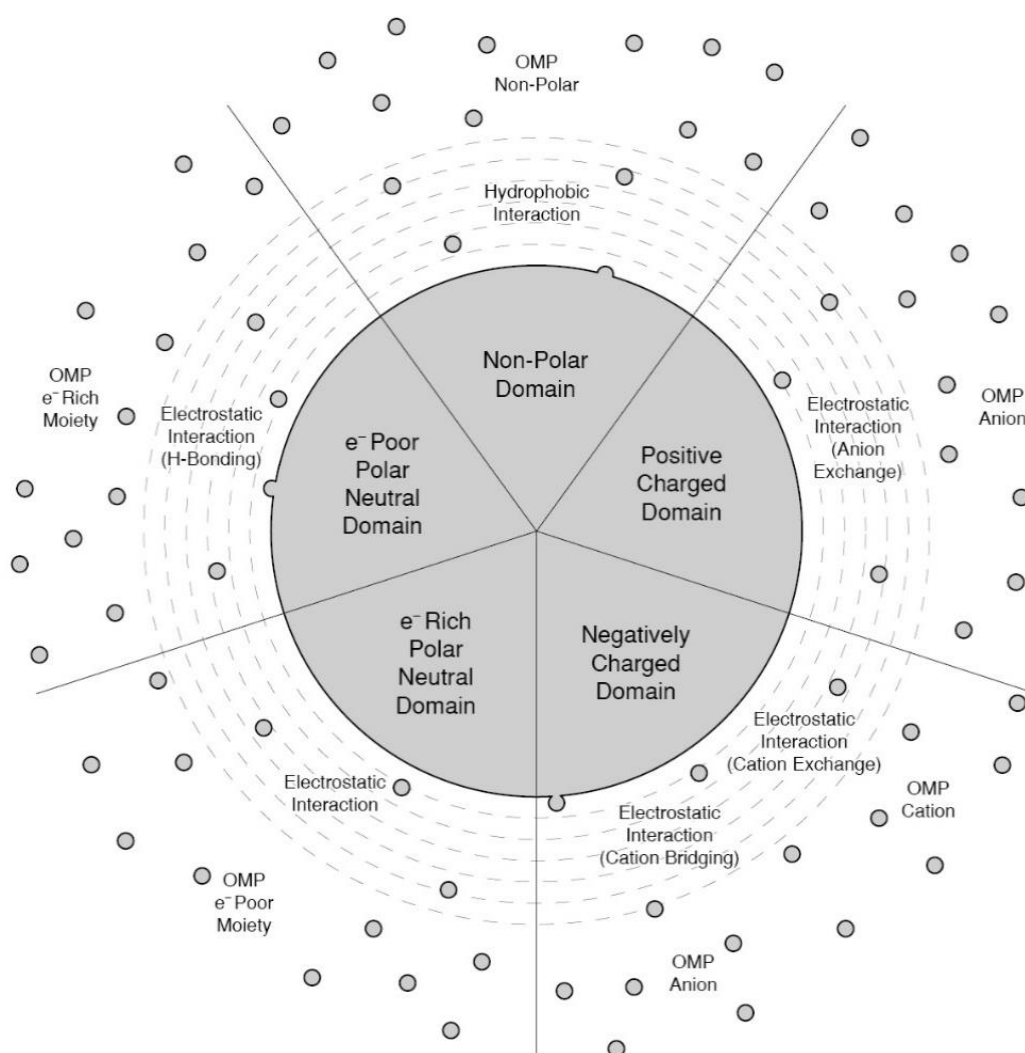


Figure 8: Schematic of the sorption of OMPs to distinct receptor sites of the biomass via multiple interaction mechanisms (adapted from MacKay and Vasudevan, 2012 and Oberoi et al., 2019).

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 are the predominant ones (Rybacka and Andersson, 2016). The liquid media characteristics, in terms of pH and ionic strength, influence 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 (Hermansson, 1999), 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 to the colloidal matter in the aqueous phase (Holbrook et al., 2004) and it is likely to influence the micropollutant distribution in the sludge (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.

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 $H < 10^{-5}$ (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 (Schwarzenbach et al., 2003). 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 into 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, and phosphoric acid esters (Neely and Blau, 1985). Chemical

oxidation and reduction, which involve the transfer of electrons from the reduced to the oxidized species, have been observed for the oxidation of halogenated solvents 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).

Partitioning of organic molecules can also occur to the foam induced by the gas during aeration (Somasundaran et al., 1972). The adsorption of a chemical to the surface of gas bubbles rising through water is a mechanism exploited to separate hydrophobic and surface-active compounds from a solution and enrich them in the generated foam (Buckley et al., 2021). This process also referred to as foam fractionation, is a well-established technology commonly applied to remove PFAS from landfill leachate (Smith et al., 2022) and contaminated groundwater (Burns et al., 2021), recover metals from slurries, and enrich proteins, phytonutrients and metabolites (Burghoff, 2012). Foam fractionation techniques have been used for the separation of a wide variety of organic molecule compounds, including cationic, anionic, and nonionic species (Somasundaran et al., 1972). However, the partitioning of pharmaceutical residuals to wastewater foam has not been researched before. In **Paper III**, evidence of the enrichment of certain pharmaceuticals in the foam generated by aeration is presented.

2.2.3. Impact of process configurations on OMP removal efficiency

The efficiency of a 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 of 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, also referred to 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 in OMP removal 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 contribute to the removal of 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). The temperature has also been observed to influence the sorption of several OMPs to 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 their solubility and the sorption capacity onto the biomass (Trapp et al., 2010).

The redox conditions impact the transformation of OMPs. The redox conditions also govern the microbial community in a biological reactor and therefore the enzyme produced. Aerobic transformation seems to be predominant compared to anaerobic degradation (Alvarino et al., 2014; Tran et al., 2013). A combination of different redox conditions has been shown to broaden the group of OMPs that are degraded (Suarez et al., 2010).

The food to microorganism ratio is likely to affect microbial activity. A lower ratio translates into a shortage of biodegradable organic matter, which could stimulate the metabolization of more persistent compounds (Park et al., 2017). High concentrations of readily biodegradable substances have been observed to suppress the biotransformation rates of some OMPs with activated sludge under nitrifying and denitrifying conditions (Su et al., 2015). The primary growth substrate can influence largely the degradation of trace organic pollutants (Çeçen and Tezel, 2017). The extent of transformation of the parent compound could be related to the degree of structural analogy between the OMPs and the primary substrate (Rieger et al., 2002). Nevertheless, the relation between the degradation of primary substrates and OMPs is complex and not fully understood. It is possible that the presence of primary substrates influences the removal kinetics and increases the OMP transformation rate. Some authors reported higher removal rates at increased loading of chemical oxygen demand (COD) and $\text{NH}_4^+\text{-N}$ (Tang et al., 2021), while others observed lower removal at higher total organic carbon (TOC) concentrations (Urase and Kikuta, 2005). Experimental results are still contradictory, and this aspect needs further investigation.

The biomass configuration, biofilm or floccular, could affect the performance of the biological reactor. Higher OMP removal rates have been observed in biofilm carriers compared to activated sludge (Falås et al., 2012). The longer biomass retention in the biofilm system promoting slow-growing organisms, together with the presence of micro-niches with different redox conditions, which enable the development of specific microbial communities, are features that possibly assist in the higher OMP removal rates (Wolff et al., 2021). In a biofilm system, the microorganisms embedded in a matrix of EPS benefit from a protection layer from inhibiting or hazardous substances (Schmidt et al., 2012). It has been shown that the presence of OMPs enhances EPS production (Pasquini et al., 2013). The composition of EPS, being a complex mixture of polymers, offers potential binding sites due to the 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; Zhang et al., 2018), and their composition in terms of protein and polysaccharides content influences the degree of partitioning (Khunjar and Love, 2011). Biofilm thickness is expected to have an impact on micropollutant removal as a result of diffusion limitation and thus substrate penetration. Thicker biofilm has also been shown to increase microbial diversity and biotransformation kinetics of several OMPs (Torresi et al., 2016).

3. Materials and methods

In all studies, a similar approach in terms of material and methods was used to answer the research questions of this thesis. The methods mainly consisted of (i) continuous-flow operation of lab-scale AGS SBR; (ii) batch experiments for biokinetics and sorption capacity assessment; (iii) full-scale sampling and (iv) characterization of the microbial community. **Paper IV** presents time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging as a method to investigate the penetration and distribution of pharmaceuticals in biofilms.

3.1. Continuous-flow operation of lab-scale AGS SBRs

Two different designs of reactors were employed. The first design (Figure 9) was operated to cultivate the granules utilized for the sorption studies (**Paper III**). To study the nutrient and OMP 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 the design of the different phases, cycle length, settling strategy, and synthetic wastewater composition 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. The second design (Figure 10) was employed for the evaluation of OMP removal during continuous-flow operations (**Paper IV** and **V**).

3.1.1. Reactor design for the cultivation of granules

Two identical lab-scale SBRs (Figure 9) were employed for the cultivation of granules for the biosorption experiments (**Paper III**). The plexiglass vessels were column-shaped open reactors with a 6 cm diameter, 132 cm 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 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, and 5 min of sludge withdrawal. The settling time was gradually decreased during the operation to allow 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 a superficial up-flow velocity of 1.5 cm s⁻¹. The dissolved oxygen (DO) and pH in the reactor were not controlled. The pH was monitored, and the data were 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 $\pm$ 3 °C. Wastewater composition and sludge inoculum are described in **Paper III**.

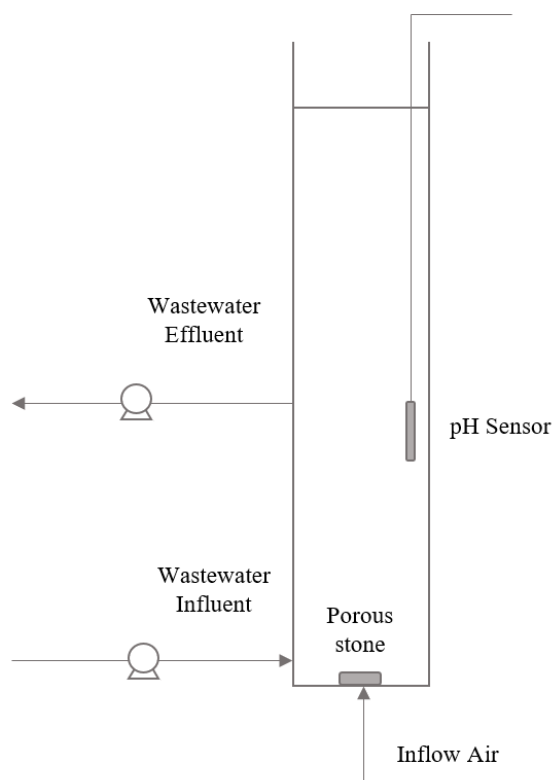


Figure 9: Schematic representation of the reactors used to cultivate granules (non-scaled image).

3.1.2. Reactor design for the removal of nutrients and OMPs

The schematic of the lab-scale AGS reactor assembled for the long-term operation experiments (**Paper V**) is illustrated in Figure 10. 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 closed system to avoid the intrusion of oxygen and to permit the monitoring of reaction products such as CO₂, CH₄, N₂, and 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 N₂ 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 7.5 ± 0.3 by the 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 of anaerobic feeding, 257 minutes of aeration, 3 minutes of settling, and 10 minutes of effluent withdrawal. The settling time was gradually decreased during the operation and the aeration phase was 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 controlled during the aeration phase. The temperature of the reactor was maintained at 21 ± 0.5 °C in a temperature-controlled room protected from sunlight. Wastewater composition and sludge inoculum are described in **Paper V**.

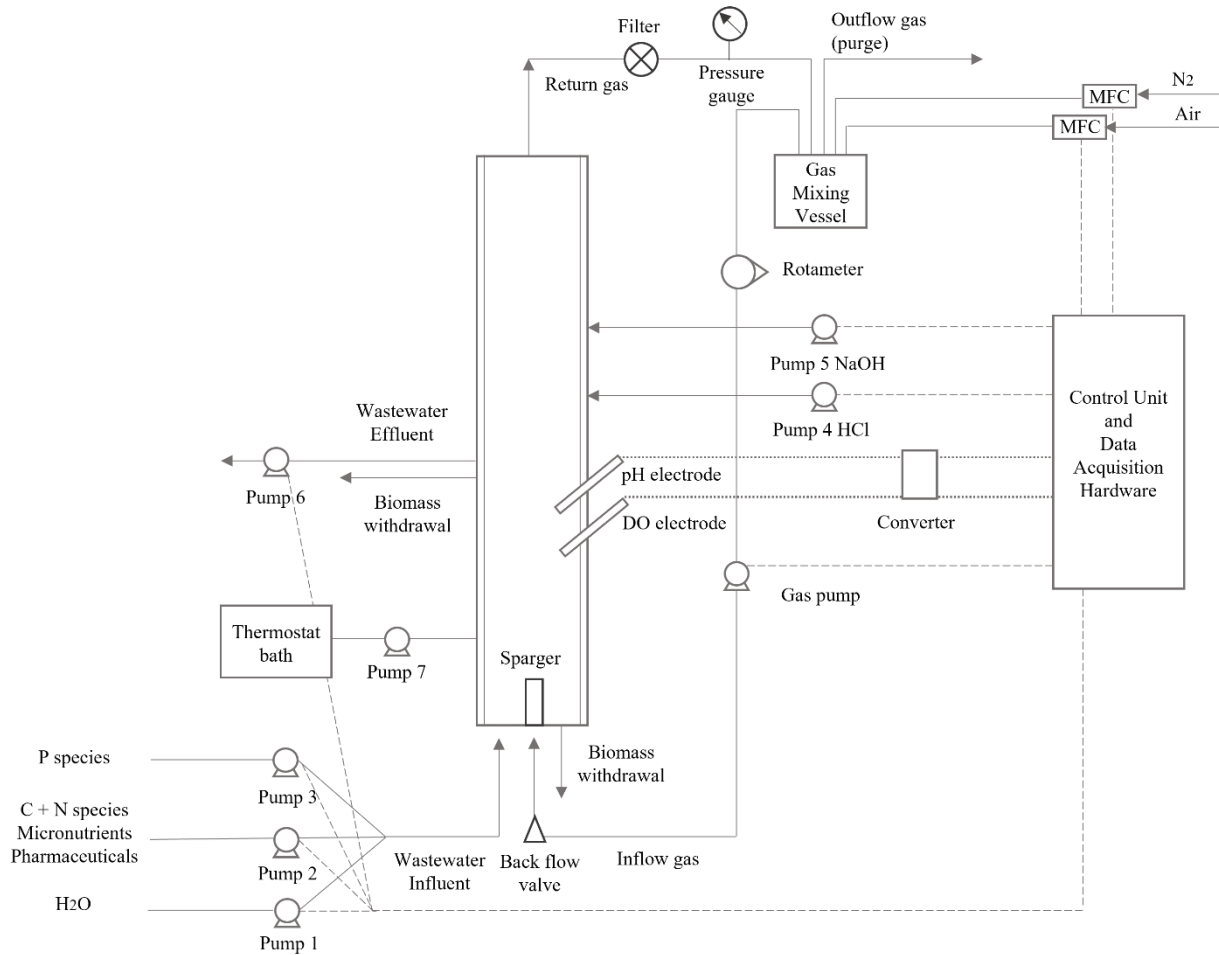


Figure 10: Schematic setup of the new AGS reactor assembled for the long-term operations of OMP removal (non-scaled image).

3.2. Batch experiments

Batch experiments were used to estimate the biokinetics of micro-pollutants removal and sorption capacity. The batches were performed separately in glass-reactors with synthetic or real wastewater spiked with reference pharmaceuticals (Table 1).

3.2.1. Biokinetics for micropollutant removal

Batch experiments were carried out with fresh biomass to determine the degradation rates of selected micropollutants. The experiments were conducted under oxic and anoxic conditions at controlled pH and temperature. Details about the transformation kinetic experiments can be found in **Paper II** and **V**. Micropollutant transformation rates were calculated according to Equation 1 assuming pseudo-first-order kinetics (Joss et al., 2006):

$$\frac{dC}{dt} = k_{bio} X_{SS} C \quad (1)$$

where C is the compound concentration ($\mu\text{g L}^{-1}$), t is the time (d), k_{bio} is the biological transformation rate constant ($\text{L gSS}^{-1} \text{d}^{-1}$), and X_{SS} is the suspended solids concentration (gSS L^{-1}). Transformation rate constants were determined through exponential regression of the measured dissolved concentration profiles and then normalized by the biomass concentration. The reaction rate constants incorporated sorption, desorption, and biological transformation. All fittings were made using least-square optimization.

3.2.2. OMP sorption batch experiments

The sorption of OMPs onto granular sludge was studied by exposing a group of pharmaceutical compounds (Table 1) to inhibited biomass. Two types of aerobic granular sludge, one dominated by fungi-like mycelia structure, and one dominated by bacterial biofilm were used. Details about the batch sorption test can be found in **Paper III**. At equilibrium, the OMP concentration sorbed onto the biomass ($C_{s,eq} \mu\text{g L}^{-1}$) is proportional to the dissolved concentration ($C_{aq,eq} \mu\text{g L}^{-1}$) and the solids-water distribution coefficient (K_d) can be expressed as (Equation 2) (Torresi et al., 2017):

$$K_d = \frac{C_{s,eq}}{C_{aq,eq} X_{biomass}} = \frac{C_{aq,0} - C_{aq,eq}}{C_{aq,eq} X_{biomass}} \quad (2)$$

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

3.3. OMP selection and analysis

The OMP selection was dependent on the laboratory technique available for the analyses. The compounds differ widely in structure and behavior and are classified according to their use (Table 1). Stock solutions of individual compounds ($100\text{--}400 \text{ mg L}^{-1}$) were prepared in ethanol

or methanol and were stored in amber glass bottles at 4 °C. The substances were obtained from Sigma Aldrich.

Table 1: Overview of the OMPs targeted in the different studies and their therapeutic class.

Class	Compound	Full-scale performances		Sorption removal	Redox conditions	Localization in AGS	DO impact
		I	II				
Analgesic	Paracetamol	X	X				
	Azithromycin		X				
Antibiotics	Sulfamethoxazole	X	X	X	X		X
	Trimethoprim	X	X	X	X		
	Ciprofloxacin	X	X	X		X	X
	Clarithromycin	X	X	X			
	Erythromycin	X		X			
Anticonvulsant	Carbamazepine	X	X	X	X	X	X
	Sertraline	X	X	X	X	X	X
Antidepressants	Citalopram	X	X	X	X	X	X
	Venlafaxine	X	X		X	X	X
Antifungal	Ketoconazole	X	X	X	X	X	
	Fluconazole	X	X	X			
Antihypertensive	Metoprolol	X	X	X	X		
	Atenolol	X	X		X		X
	Losartan	X	X	X	X	X	
	Propranolol	X	X				
Antineoplastic	Methotrexate	X	X	X	X		
Diuretic	Furosemide	X	X				
Hormones	β-Estradiol			X			
	17α-Ethinylestradiol			X			
	Levonorgestrel			X			
	Estrone	X	X				
Insecticide	Imidacloprid	X					
	Acetamiprid	X					
Non-steroidal anti-inflammatory	Diclofenac	X	X	X	X	X	
	Naproxen	X	X	X	X		
	Ibuprofen	X	X				
	Ketoprofen						X
	Mefenamic acid						X
Opiate analgesic	Tramadol	X	X	X	X		
Plasticizer	Bisphenol A	X	X				
Sedative hypnotic	Oxazepam	X	X	X	X		
	Zolpidem	X	X	X	X		

Samples from micropollutant analysis were analyzed with (i) ultra-high performance liquid chromatography - tandem mass spectrometry (UPLC MS/MS), and (ii) high performance liquid chromatography (HPLC) MS/MS techniques. Information about internal standards, MS/MS technique and methods, limits of detection and quantification are described in **Papers II, III, and V**.

3.4. Full-scale sampling

The presence and removal of OMPs were investigated in two Swedish full-scale WWTPs designed for nutrient removal. Influent and effluent samples of the investigated biological reactors were analyzed to evaluate OMP removal efficiencies.

The removal efficiency of each OMP was calculated by comparing the concentration in the influent samples and the corresponding effluent samples (Equation 3):

$$\text{Removal [\%]} = \frac{(C_{\text{influent}} - C_{\text{effluent}})}{C_{\text{influent}}} \cdot 100 \quad (3)$$

3.4.1. Rya WWTP

In **Paper I**, the presence and removal of target OMPs were investigated at the Rya WWTP, which treats wastewater from 970 000 Population Equivalent (PE) in the Gothenburg region (Sweden). Wastewater treatment includes primary settling, chemical removal of phosphorus, and biological nitrogen removal. The WWTP consists of several biological treatment processes, including an activated sludge reactor for denitrification and organic matter removal, nitrifying trickling filters, nitrifying moving bed bioreactors (MBBRs), and post-denitrifying MBBRs (Figure 11). The OMP sampling was performed over two separate summer campaigns and grab samples were collected at the influent and effluent of the different biological reactors.

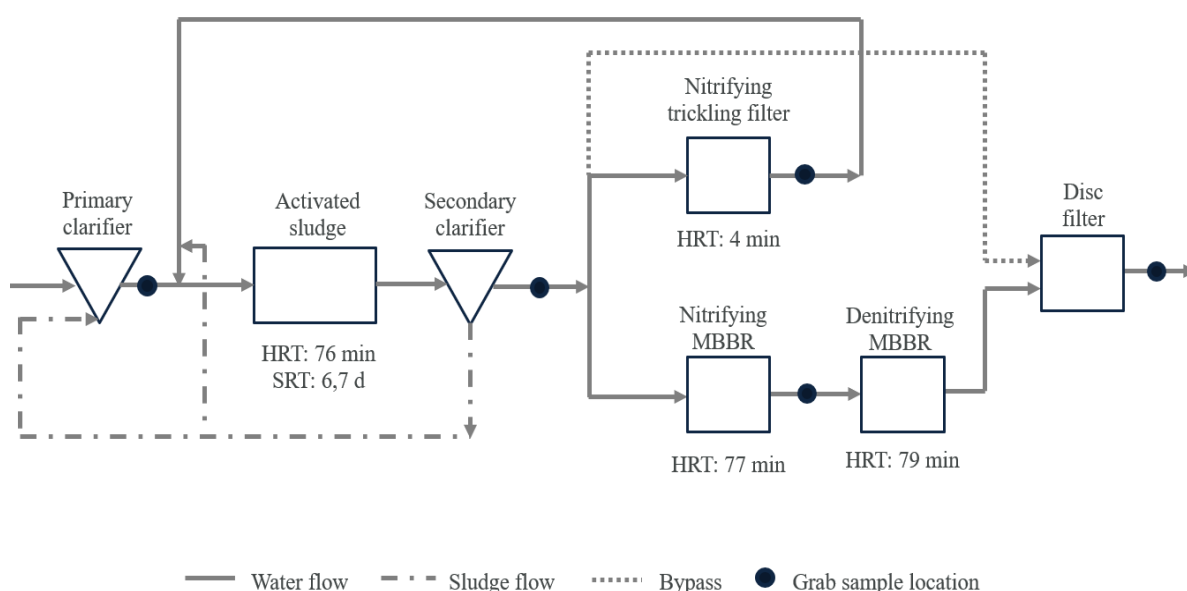


Figure 11: Process scheme, retention times, and sampling location at the full-scale WWTP located in Gothenburg.

3.4.2. Österröd WWTP

The Österröd WWTP operated by the municipality of Strömstad, Sweden, was investigated in **Paper II**. The plant receives predominantly domestic wastewater and is designed for 30 000 PE. The existing WWTP consists of a CAS process, operated in parallel with a Nereda® plant (Nereda® is a trademark owned by Royal HaskoningDHV) consisting of two AGS reactors for organic matter and nutrient (nitrogen and phosphorus) removal (Figure 12). The AGS reactors treat 60% (distributed equally) of the total influent flow received during dry weather flow, and the CAS treats the remaining 40%. The AGS process has a semi-continuous flow mode and is operated as sequencing batch reactors (SBRs), consisting of a simultaneous feeding and effluent withdrawal period, a reaction period, and a settling and sludge withdrawal phase. The CAS process has a continuous flow mode and is operated with both pre- and post-denitrification as a series of seven continuous stirred-tank reactors (CSTRs). The sampling campaign was conducted in the winter months for two consecutive weeks (November 15th – November 26th, 2020). Samples were collected as 24 h flow proportional samples from the influent and effluent of the biological units. The effluents of SBR1 and SBR2 were combined for the analysis of OMPs and the calculation of AGS removal performances.

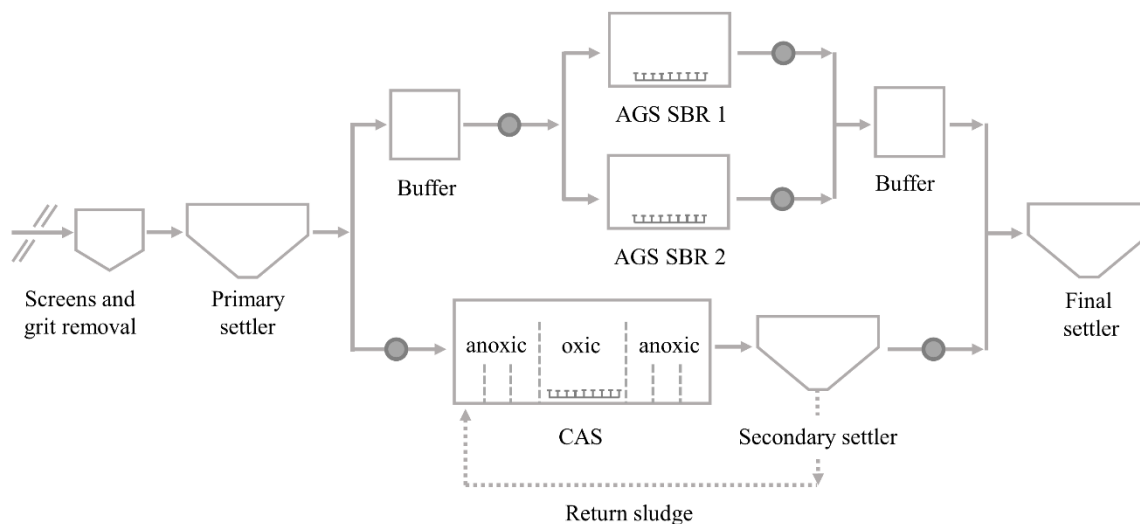


Figure 12: Schematic diagram showing the treatment process in the Österröd WWTP and the sampling points (grey circles).

3.5. Microbial characterization

Microbial characterization was investigated by the means of metagenomic analysis through next generation Illumina sequencing. The results from the microbial characterization were used for (i) evaluating the most abundant taxonomic affiliations in the investigated biological reactors (**Paper II** and **III**), (ii) comparing gene diversity between the conventional activated sludge and the aerobic granular sludge, and (iii) investigating the resistome profiles (**Paper II**). Details about DNA extraction protocol, library preparation, and sequencing instruments can be found in the attached papers.

3.6. SIMS imaging in biofilm

ToF-SIMS imaging was explored in **Paper IV** as a method to investigate the localization and distribution of eight pharmaceuticals (Table 1) within the biofilm of a lab-scale cultivated AGS. Pharmaceutical standard powders were mounted onto indium sheets for ToF-SIMS analysis. Granules used in the analysis were harvested from the reactor. One batch of granules was rinsed with deionized water, and stored at -80 °C, to serve as control biofilms without pharmaceutical exposure for ToF-SIMS analysis. Another batch of granules was exposed to a cocktail of the selected OMPs. Filtered effluent wastewater from the reactor was spiked with micropollutants at the start of the experiment at a concentration of 10 mg L⁻¹. The batch was aerated continuously, and the granules were sampled for SIMS analysis after six hours of exposure to OMPs, rinsed with deionized water, and stored at -80 °C. To illustrate the distribution of pharmaceuticals inside the granules, biofilm sections (12 µm thickness) were obtained using a microtome cryostat at -20 °C.

The ToF-SIMS surface analysis was carried out using a ToF-SIMS 5 instrument (ION-ToF GmbH, Münster, Germany) equipped with a Bi cluster ion gun as a primary ion source. Mass spectra in positive ion mode were acquired by using a 25keV Bi₃⁺ primary ion source. Control and treated biofilm spectra were compared and analyzed. The ion peaks *m/z* that appeared in the treated but not in the control were identified. Possible assignments for the identified peak were performed mainly using the standard pharmaceuticals mass spectrum analyzed separately and by employing the defined mass spectra from previous studies.

3.7. Analytical methods

The AGS SBR performances were investigated by analyzing the dissolved organic carbon, nitrogen, and phosphorus content of aqueous samples. Dissolved organic carbon and nitrogen were measured with a TOC/total N analyzer (Shimadzu) after filtration through a polyethersulfone membrane syringe filter of 0.2 µm pore size. Cations (NH₄⁺-N) and anions (NO₃⁻-N, NO₂⁻-N, PO₄³⁻-P) were analyzed with an Ion Chromatograph (Dionex ICS-900).

Physical sludge parameters, such as suspended solids (SS), volatile suspended solids (VSS), and sludge volume index (SVI) were quantified using standard methods (APHA, 2005). Sludge morphology was observed by light microscopy (Olympus BX53) equipped with a digital camera (Olympus DP11) and by an environmental scanning electron microscopy (ESEM, FEI Quanta200 FEG-ESEM).

4. Results and Discussion

4.1. Occurrence of OMPs in urban wastewater and full-scale removal efficiencies

All measured OMPs were detected in the primary clarified wastewater at concentrations ranging from 0.1 ng L⁻¹ to 48 µg L⁻¹ (Figure 13) (**Paper I** and **II**). These values are in accordance with the concentrations detected and compiled by previous studies investigating micropollutant levels in Swedish wastewater (Falås et al., 2012a; Paxéus, 2004; Zorita et al., 2009). Only few of the investigated substances showed an average influent concentration > 1 µg L⁻¹, namely ibuprofen, paracetamol, naproxen, and losartan. Among the investigated OMPs at the full-scale WWTPs in Göteborg and Strömstad, the removal efficiencies in the biological units varied greatly (Figure 14 and Figure 15). The term removal refers to all the losses of the parent compounds due to different physicochemical and biological mechanisms (sorption to solid matter, and abiotic and microbially mediated transformation). Ibuprofen, paracetamol, and naproxen, which are frequently used anti-inflammatory and analgesic substances, were readily transformed in the biological treatments. The antimycotic ketoconazole showed consistently high elimination (> 90%) from the water phase at both WWTPs. High reduction (> 70%) was also observed for compounds such as bisphenol A, ciprofloxacin, estrone, and methotrexate. Atenolol, losartan, sertraline, and sulfamethoxazole showed significant removal (40-70%). Sorption to biomass rather than biodegradation, was likely the main mechanism for a few OMPs, like ciprofloxacin, ketoconazole, and sertraline (Lajeunesse et al., 2012; Lindberg et al., 2010, 2005; Svahn and Björklund, 2019). Partial or no removal (< 25%) was obtained for the most persistent OMPs, such as carbamazepine, fluconazole, furosemide, metoprolol, oxazepam, propranolol, and tramadol, which showed resistance to microbial degradation by either activated sludge or aerobic granular sludge treatments.

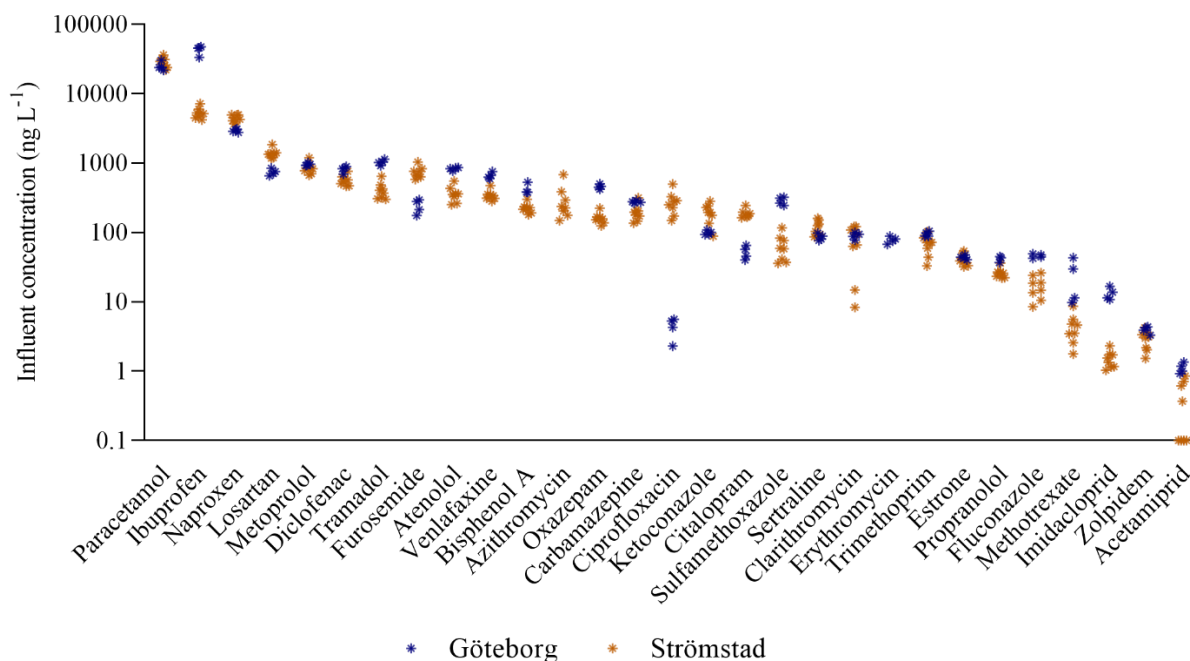


Figure 13: Incoming OMP concentrations in influent wastewater after primary settler at the WWTPs in Göteborg and Strömstad (**Paper I** and **II**). Each point represents an observation.

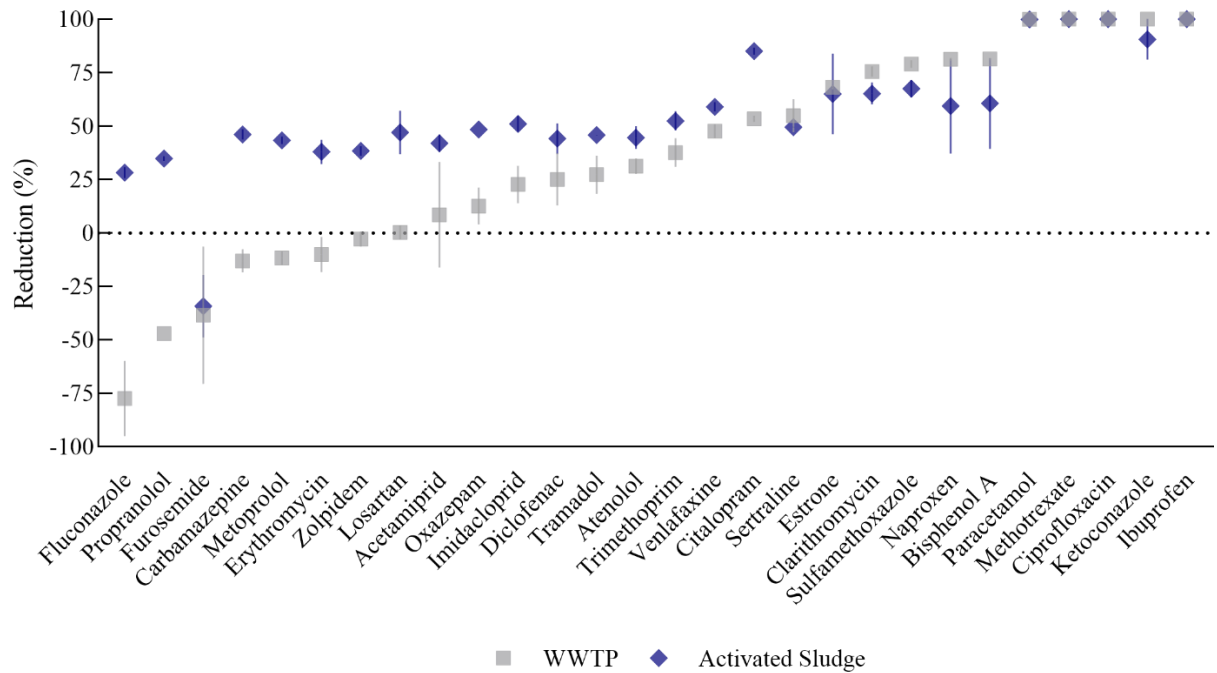


Figure 14: Comparison of average removal efficiencies of the Activated Sludge unit and the entire WWTP in Göteborg (**Paper I**). The bars indicate standard deviations (n=4).

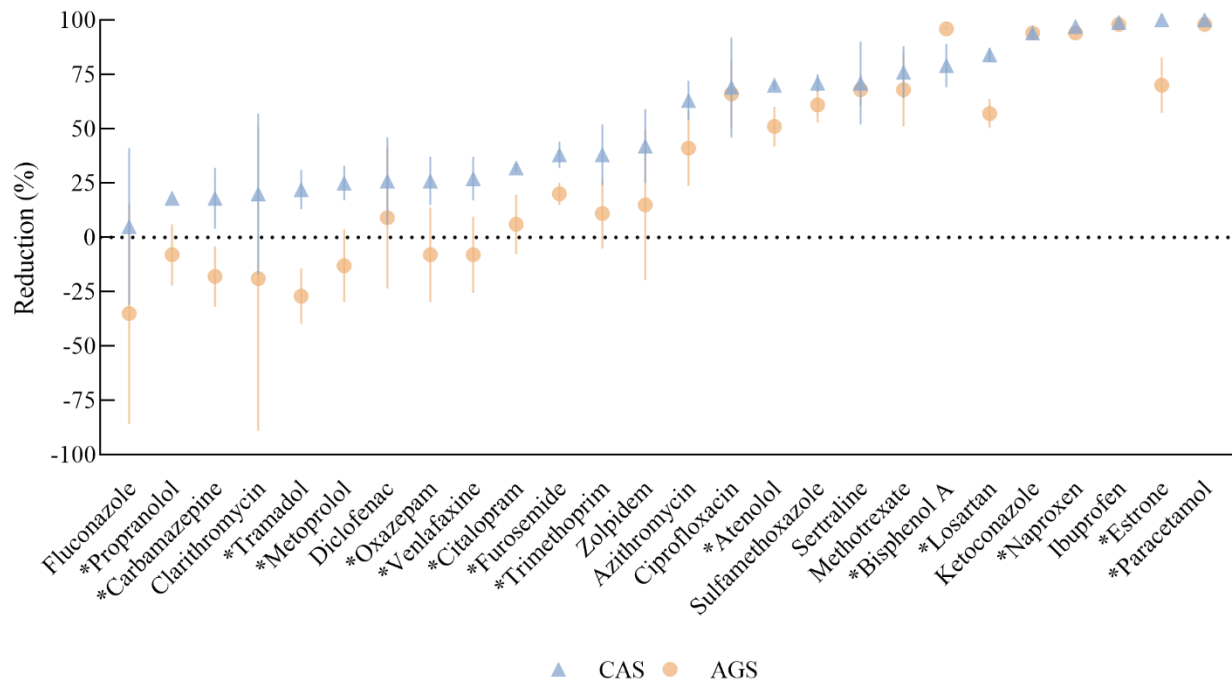


Figure 15: Average removal efficiencies of the conventional activated sludge (CAS) and the aerobic granular sludge reactors (AGS) in Strömstad WWTP (**Paper II**). Bars represent standard deviations (n=4). The asterisk refers to compounds that have residual fractions significantly different between the two biomass systems (t-test, p value < 0.05).

Low or negative removal efficiencies for these OMPs have been observed in several previous studies, indicating their persistence through biological processes (Ashfaq et al., 2017; Falås et al., 2012a; Kim and Oh, 2020; Leiviskä and Risteelä, 2021; Lindberg et al., 2010; Peng et al., 2012). The negative reduction was reported for compounds with higher concentrations in the effluent than 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 (Leiviskä and Risteelä, 2021; Verlicchi et al., 2012). At the lower concentration range found for some OMPs, the associated uncertainties are high. Instrumental errors might lead to a higher quantification in the effluent than in the influent samples (Verlicchi et al., 2012). Negative removals can also be explained by sample collections that do not account for the process HRT (Zorita et al., 2009). While for some OMPs negative reduction could arise from either the biological transformation of the deconjugated compounds or the release of compounds sorbed onto the particulate after biological treatment, for other substances further investigation is required (Verlicchi et al., 2012). In **Paper III**, measurements of pharmaceuticals in the foam generated by gas sparging suggest that enrichment in the foam is a mechanism that might affect several OMPs. Since aeration is a common feature in wastewater treatment, investigations are needed to quantitatively determine whether pharmaceuticals could be enriched in the foam commonly present on top of biological reactors. If the magnitude of this mechanism in full-scale systems is environmentally relevant, it may have important implications for sampling approaches when determining pharmaceutical concentrations in wastewater.

At the WWTP located in Göteborg, the conventional activated sludge reactor, being the first in line, contributed to most of the removal from the water phase. Additional removal of a few compounds was observed in the biofilm units, but most of the persistent compounds remained stable through all biological treatments.

4.2. Pharmaceutical removal with AGS and CAS - a comparison

4.2.1. Full-scale performances

For all OMPs investigated at Österröd WWTP but one (bisphenol A), the removal performances were generally higher in the CAS process than in the AGS process (Figure 15). Since the two systems received the same wastewater, influent characteristics were ruled out as a cause of the performance difference.

The different removal efficiencies obtained in the two reactor types at the full-scale WWTP can be attributed to several reasons, including (i) microbial community compositions and metabolic activity; (ii) the size and the shape of flocs and granules (Figure 3a and b) influencing the exposure of OMPs to the biomass; (iii) the HRTs in the two processes resulting in longer contact time between the activated sludge and the OMPs to be transformed (11.4 hours in the AGS system and 16.2 hours in the CAS reactor, Table 2 in **Paper II**).

No significant differences were observed between AGS and CAS for compounds having low residual fractions attributed to sorption mechanisms, such as ciprofloxacin, ketoconazole, and sertraline. Although AGS might have a lower specific surface area due to the size of this biofilm, the higher biomass concentration in the tanks provided a comparably similar surface

area to the CAS system. Moreover, the concentrations of OMPs were relatively low and the biomass surface area might not be a limiting factor in biological sorption processes.

4.2.2. Batch experiments

To evaluate the extent of microbial degradation of OMPs in AGS and CAS, batch experiments were performed over 48 hours on a selection of the OMPs analyzed in the full-scale WWTP (**Paper II**). First-order degradation kinetics was fitted to all compounds, except for two, and one transformation rate constant k_{bio} was assumed to be valid over the whole experiment (one phase decay). The concentrations of the antimycotic ketoconazole (neutral and hydrophobic compound at circumneutral pH) and the selective serotonin reuptake inhibitor sertraline (protonated and moderately hydrophobic molecule at circumneutral pH) dropped considerably after ten minutes of incubation, indicating removal predominantly by sorption. In Figure 16, the biotransformation kinetics under oxic conditions are presented for three representative compounds out of the 16 pharmaceuticals analyzed (for the remaining compounds, refer to **Paper II**). Generally, most of the compounds showed similar transformation rates between the two types of biological systems (e.g., zolpidem) or higher transformation rates with the floccular sludge from the CAS tank (i.e., atenolol). However, only one compound (diclofenac) had faster removal with AGS biomass and was hardly removed by CAS. Biofilm from MBBR carriers has previously been observed to transform diclofenac at a significantly higher rate than activated sludge, which supports this observation (Falås et al., 2013; Jewell et al., 2016; Wolff et al., 2021).

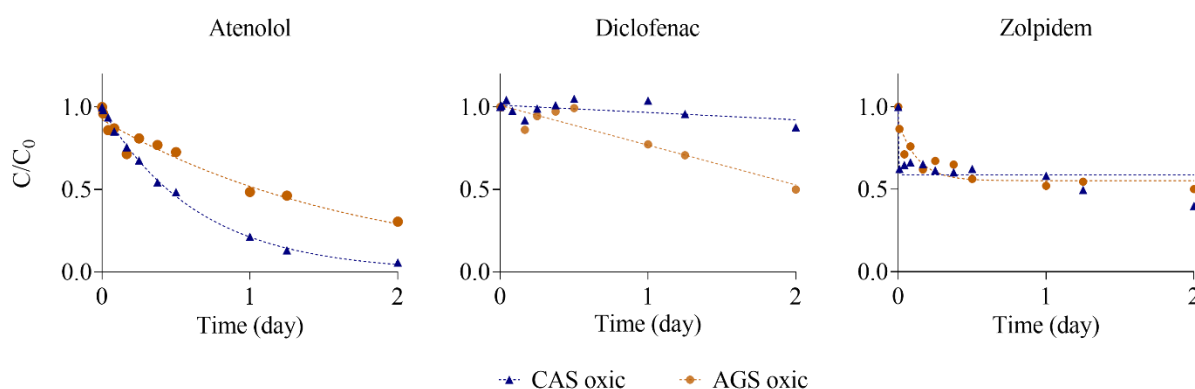


Figure 16: Residual fractions of three pharmaceuticals in incubations under oxic conditions with CAS and AGS (adapted from **Paper II**). The lines represent the first-order degradation kinetics.

The underlying mechanisms for the OMP removal difference between floccular and granular sludge are not clear but could be associated with the microbial communities and the mass transfer limitations of the biofilm system. In the granules, which can be regarded as free-floating biofilms, microorganisms are not equally distributed. Some groups are more abundant at the surface of the biofilm and others are more prevalent in the biofilm interior (Wilén et al., 2018). There is yet no information available on where the transformation of OMPs takes place, i.e., outer biofilm layer or floc surface, inner biofilm layer, or bulk medium (Joss et al., 2004). It might be speculated that mass transfer is a limiting parameter in the removal of micropollutants in AGS. Biofilm processes are governed by diffusion processes and in most biofilm systems, the substrate uptake rate is limited by mass transfer (Siegrist and Gujer, 1985). Because of the dense and thick structure of the granules, the diffusive transport of substrates

from the bulk liquid into the biofilm can be relatively slow compared to the volumetric reaction rates (van den Berg et al., 2020). The diffusion of substrates into a biofilm is dependent on the biomass density and decreases with increasing biofilm density (Horn and Morgenroth, 2006). It is therefore likely that the differences in CAS and AGS conformation and density played a role in the diffusion of OMPs in the biomass and their removal performances. Whether diffusion rate or enzymatic reactivity is the rate-limiting step for the transformation process of OMP is still a fundamental question yet to be answered (Wei et al., 2019).

4.2.3. Microbial communities

The metagenomes retrieved from the biomass of the CAS and the two AGS reactors on four occasions revealed differences in biodiversity among the reactors (**Paper II**). The AGS samples had on average $9 \pm 6\%$ higher gene cluster diversity than the CAS samples ($p < 0.05$, ANOVA and Tukey's HSD, $n=4$), but there was no significant difference between the samples from the two AGS reactors (Figure 17a). Furthermore, the gene cluster composition of the AGS and CAS metagenomes were different ($p < 0.01$, permanova), but the two AGS reactors were not. This was visualized using principal coordinate analysis (PCoA) (Figure 17b). There was a clear separation between AGS and CAS samples along the first principal coordinate. This could likely be explained by the different operational conditions and structural differences (size, micro-niches with different redox conditions) of AGS and CAS since both systems received the same wastewater with the same seasonal variations. The seasonal changes and the transition to the winter months resulted in a clear shift of the gene cluster composition as described by the second coordinate.

AGS had higher gene diversity compared to the CAS system on all sampling occasions. Taxonomic and functional biodiversity has been positively associated with the rates of some micropollutant biotransformations (Johnson et al., 2015a; Stadler et al., 2018; Stadler and Love, 2016; Torresi et al., 2016). The hypothesis usually formulated is that diverse microbial communities are likely to have a versatile metabolic potential and/or more functional traits that give them the potential to transform OMPs via enzymatic degradation processes (Stadler et al., 2018; Wolff et al., 2018). In this study, AGS showed lower potential for OMP transformation than CAS in batch tests as well as in full-scale, despite having a $9 \pm 6\%$ higher gene cluster diversity. Because of the small difference in diversity between AGS and CAS in this study, other factors, such as microbial community composition, micropollutant exposure to biomass, or mass transfer limitations, may have played more important roles in the differing OMP removal efficiencies.

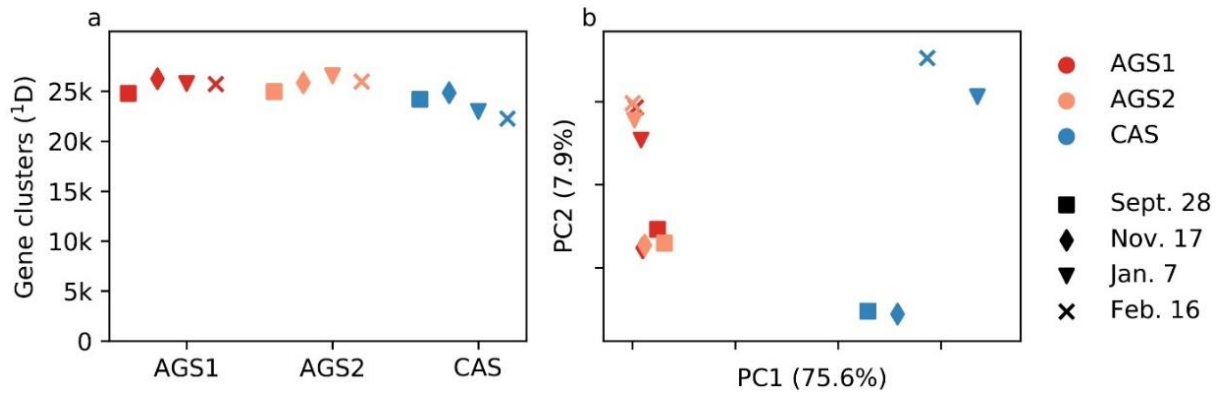


Figure 17: Gene cluster diversity (a) and principal coordinate analysis (PCoA) of dissimilarity in gene cluster composition between samples (b).

The most abundant taxonomic affiliations in the metagenomes are shown in Figure 18. *Actinobacteria*, *Proteobacteria*, *Nitrospirae*, and *Bacteroidetes* phyla predominated in both AGS and CAS. *Chloroflexi* was also found among the abundant phyla in CAS. The most abundant genera in the biomass samples were characterized as bacteria responsible for phosphorus removal (e.g., *Candidatus* (*Ca.*) *Microthrix*, *Dechloromonas*, *Ca. Accumulibacter*), as well as for nitrogen conversion (e.g., *Nitrospira*, *Rhodoferrax*, *Ca. Nitrotoga*, *Sulfuritalea*), and COD removal (e.g., *Rubrivivax*) (Albertsen et al., 2016; Cydzik-Kwiatkowska and Zielińska, 2016; Li et al., 2020; Yu and Zhang, 2012). The genus *Propionivibrio*, which harbors glycogen accumulating organisms (GAOs) (Albertsen et al., 2016), was also common in all reactors, with decreasing abundances in the colder seasons. *Ca. Competibacter*, which is also a GAO, was primarily detected in the AGS biomass. The genus *Flavobacterium*, which includes species that can support granule formation and promote the production of EPS (Simonsen Dueholm et al., 2021), was present in all the reactors with higher abundance in the AGS than the CAS. In contrast to the AGS systems, CAS was dominated by genera with filamentous organisms, such as *Ca. Microthrix*, known to be responsible for bulking and foaming in nutrient removal plants (Simonsen Dueholm et al., 2021), and *Ca. Promineofilum* (Speirs et al., 2019). *Nitrospira*, important for ammonium and nitrite oxidation (Spasov et al., 2020), was the second most abundant genus in the CAS and was considerably higher than in the AGS. *Nitrospira* has been found to be positively correlated with the removal of some OMPs (Wolff et al., 2021). *Ca. Nitrotoga*, a widespread nitrite-oxidizing bacterium in cold and moderate climates (Spieck et al., 2021), was abundant in the AGS samples, but hardly observed in the CAS samples. Similarly, carbon storing organisms, such as *Dechloromonas*, *Ca. Accumulibacter*, *Propionivibrio*, and *Ca. Competibacter* were more prevalent in the AGS biomass.

<i>Proteobacteria; Caedimonas</i>	<0.1	<0.1	<0.1	<0.1		<0.1	<0.1	<0.1	<0.1		0.6	17	0.1	<0.1
<i>Chloroflexi; CandidatusPromineofilum</i>	<0.1	<0.1	<0.1	<0.1		<0.1	<0.1	<0.1	<0.1		19	11	14	0.8
<i>Proteobacteria; CandidatusCompetibacter</i>	0.9	0.9	1.7	0.8		0.6	0.6	1.1	0.5		<0.1	<0.1	<0.1	<0.1
<i>Proteobacteria; Polaromonas</i>	0.6	0.7	0.9	1.0		0.6	0.7	0.8	1.1		0.6	1.0	0.7	1.3
<i>Proteobacteria; Novosphingobium</i>	0.8	0.9	1.2	1.1		0.8	1.0	1.1	1.0		0.7	0.7	0.5	0.4
<i>Proteobacteria; Simplicispira</i>	0.7	1.0	0.9	1.0		0.9	1.1	0.8	0.8		2.0	1.9	1.2	2.1
<i>Proteobacteria; Sphingobium</i>	1.0	1.3	2.0	2.0		0.9	1.3	1.9	1.9		0.6	0.5	0.5	0.5
<i>Proteobacteria; Sphaerotilus</i>	0.2	5.1	0.9	0.9		0.2	1.6	2.8	1.4		0.6	0.9	0.4	0.4
<i>Proteobacteria; Rhodobacter</i>	1.0	1.2	1.5	1.6		1.0	1.6	1.9	1.9		0.8	1.1	1.3	1.1
<i>Proteobacteria; Sulfuritalea</i>	1.5	1.3	1.2	1.5		1.3	1.1	1.2	1.3		2.9	2.6	0.7	1.0
<i>Proteobacteria; CandidatusNitrotoga</i>	2.3	2.1	3.0	3.1		1.8	2.1	3.1	2.9		0.1	<0.1	<0.1	<0.1
<i>Proteobacteria; Ideonella</i>	2.1	2.0	2.3	2.2		1.9	2.0	2.1	2.3		1.3	1.3	1.0	1.4
<i>Proteobacteria; Rhodoferax</i>	1.0	1.3	1.8	2.2		1.1	1.4	1.5	2.3		1.2	2.8	2.2	3.6
<i>Proteobacteria; Rubrivivax</i>	3.0	2.3	2.2	2.2		2.7	2.2	1.9	2.1		1.0	1.3	1.1	1.6
<i>Bacteroidetes; Flavobacterium</i>	3.2	3.0	2.8	2.7		2.2	1.9	2.3	2.3		1.4	1.3	1.1	1.1
<i>Proteobacteria; Propionivibrio</i>	5.6	4.4	2.2	1.8		5.5	4.3	2.1	2.2		3.1	2.8	0.5	0.6
<i>Proteobacteria; CandidatusAccumulibacter</i>	6.0	5.7	1.7	1.4		5.5	6.0	1.7	1.9		2.7	1.9	0.4	0.5
<i>Nitrospirae; Nitrospira</i>	1.4	1.4	2.2	1.9		0.7	1.0	1.5	1.1		9.1	7.3	7.6	5.1
<i>Proteobacteria; Dechloromonas</i>	4.3	4.3	1.6	1.6		7.7	7.3	2.0	2.9		3.6	4.6	1.0	1.6
<i>Actinobacteria; CandidatusMicrothrix</i>	5.1	2.8	2.0	1.6		5.7	4.2	2.2	1.6		15	14	33	33
	Sept28_AGS1	Nov17_AGS1	Jan7_AGS1	Feb16_AGS1		Sept28_AGS2	Nov17_AGS2	Jan7_AGS2	Feb16_AGS2		Sept28_AS	Nov17_AS	Jan7_AS	Feb16_AS

Figure 18: Relative abundance (%) of the most abundant taxonomic classifications (phylum; genus) of the genes in the samples. To keep consistency with the published work (**Paper II**), the phylum names in the figure and the text are not updated according to the newly renamed prokaryote phyla by the International Committee on Systematics of Prokaryotes (Oren and Garrity, 2021).

The potential to transform OMPs in biological processes seems to be influenced predominantly by the process conditions and the microbial communities (Helbling et al., 2015; Wolff et al., 2018). It remains a challenge to predict the correlation between the OMP degradation rates and taxonomic composition. Some microorganisms have been suggested as biological indicators for the improved transformation of OMPs. For the genera *Acidibacter*, *Nitrospira*, and *Rhizomicrobium*, a significant link between their relative abundance and the transformation rates of some OMPs was observed (Wolff et al., 2021). Even microorganisms with low relative abundance (<1% of the biomass) could play an important role in the degradation of certain micropollutants (Escolà Casas et al., 2017; Vuono et al., 2016).

4.3. Impact of operating conditions on OMP transformation rates

4.3.1. Redox conditions

The transformation rates of selected OMPs exposed to AGS and CAS were compared between oxic and anoxic conditions. In Figure 19, the kinetics under oxic and anoxic conditions are presented for three representative compounds (for the remaining compounds, refer to **Paper II**). Table 2 summarizes the transformation rate constants obtained in the biological degradation experiments normalized to the biomass concentration.

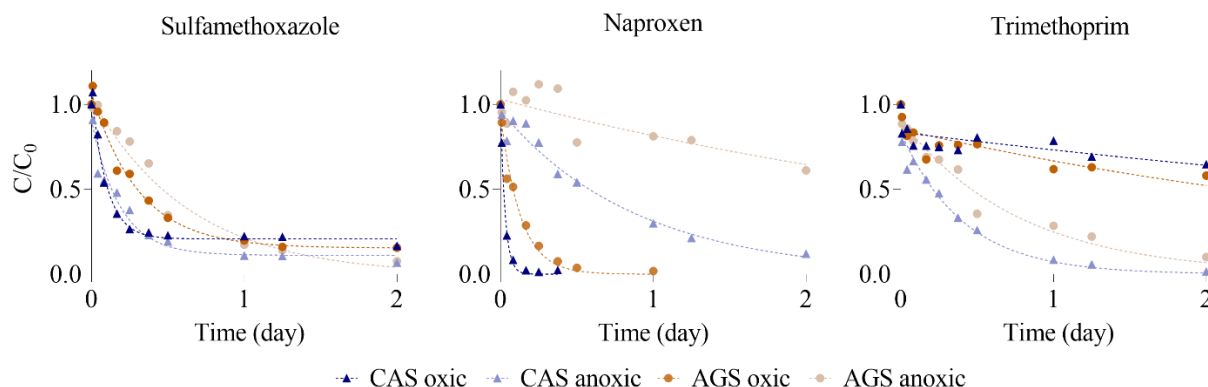


Figure 19: Residual fractions of three representative pharmaceuticals after incubations under oxic and anoxic conditions with CAS and AGS (adapted from **Paper II**). The lines represent the first-order degradation kinetics.

The availability of different electron acceptors, i.e., oxygen and nitrate, affected the transformation rate of OMPs. Some compounds (e.g., atenolol, citalopram, and sulfamethoxazole) were transformed at both oxic and anoxic conditions. Higher reaction rates (k_{bio} differences $> 0.04 \text{ L gSS}^{-1} \text{ d}^{-1}$) were obtained at oxic than anoxic conditions for losartan, naproxen, sulfamethoxazole, and zolpidem with both AGS and CAS. Similar findings have been observed in previous studies (Edefell et al., 2021a, 2021b; Falås et al., 2013; Suarez et al., 2010). However, atenolol and trimethoprim reaction rates were lower at oxic than anoxic conditions (k_{bio} differences $> 0.04 \text{ L gSS}^{-1} \text{ d}^{-1}$) with both AGS and CAS biomass types. Metoprolol showed faster transformation under anoxic conditions with AGS, but a higher rate under oxic conditions with CAS. The hypnotic zolpidem showed recalcitrant behavior under anoxic conditions with both biomasses.

Results and Discussion

Table 2: Biological transformation rate constants (k_{bio}) normalized to biomass concentration under oxic and anoxic conditions. The 95% confidence intervals are indicated by $\pm$. Rate constants of $0.04 \text{ L gSS}^{-1} \text{ d}^{-1}$ correspond to the limit of experimental resolution in this study.

Compound	$k_{bio} (\text{L gSS}^{-1} \text{ d}^{-1})$			
	CAS oxic	CAS anoxic	AGS oxic	AGS anoxic
Naproxen	$11.16^* \pm 1.55$	0.37 ± 0.09	$2.55^* \pm 0.64$	0.08 ± 0.05
Sulfamethoxazole	1.68 ± 0.91	1.21 ± 0.48	0.72 ± 0.21	0.52 ± 0.12
Losartan	0.77 ± 0.24	0.07 ± 0.05	0.18 ± 0.03	0.07 ± 0.04
Atenolol	0.51 ± 0.03	0.62 ± 0.13	0.19 ± 0.05	0.56 ± 0.11
Metoprolol	0.15 ± 0.02	0.09 ± 0.05	≤ 0.04	0.13 ± 0.05
Zolpidem	0.10 ± 0.08	≤ 0.04	0.11 ± 0.08	≤ 0.04
Citalopram	0.07 ± 0.09	0.09 ± 0.11	0.09 ± 0.09	0.13 ± 0.14
Trimethoprim	≤ 0.04	0.81 ± 0.26	0.08 ± 0.05	0.43 ± 0.10
Diclofenac	≤ 0.04	≤ 0.04	0.10 ± 0.03	≤ 0.04
Carbamazepine	≤ 0.04	≤ 0.04	≤ 0.04	≤ 0.04
Fluconazole	≤ 0.04	≤ 0.04	≤ 0.04	≤ 0.04
Oxazepam	≤ 0.04	≤ 0.04	≤ 0.04	≤ 0.04
Tramadol	≤ 0.04	≤ 0.04	≤ 0.04	≤ 0.04
Venlafaxine	≤ 0.04	≤ 0.04	≤ 0.04	≤ 0.04

* Removed beyond LOQ before the end of the experiments. Transformation rates estimated with fewer data points. The line – represents compounds that do not follow first-order kinetics.

4.3.2. Dissolved oxygen

In **Paper V** the impact of dissolved oxygen on the removal efficiencies of nutrients and pharmaceuticals by AGS was investigated in a lab-scale reactor. To evaluate the extent of microbial transformation of pharmaceuticals by AGS adapted at two different DO levels, batch experiments were performed over 24 hours under oxic conditions. In Figure 20 the kinetics are presented for three representative compounds (for the remaining compounds, refer to **Paper V**). Phase I refers to the transformation kinetics of OMPs with AGS adapted to an oxygen concentration of $1.8 \text{ mg O}_2 \text{ L}^{-1}$, while Phase II denotes the removal with AGS adapted to an oxygen level of $4.5 \text{ mg O}_2 \text{ L}^{-1}$. First-order degradation kinetics was fitted to all compounds, except for sertraline, which was likely removed by sorption mechanisms.

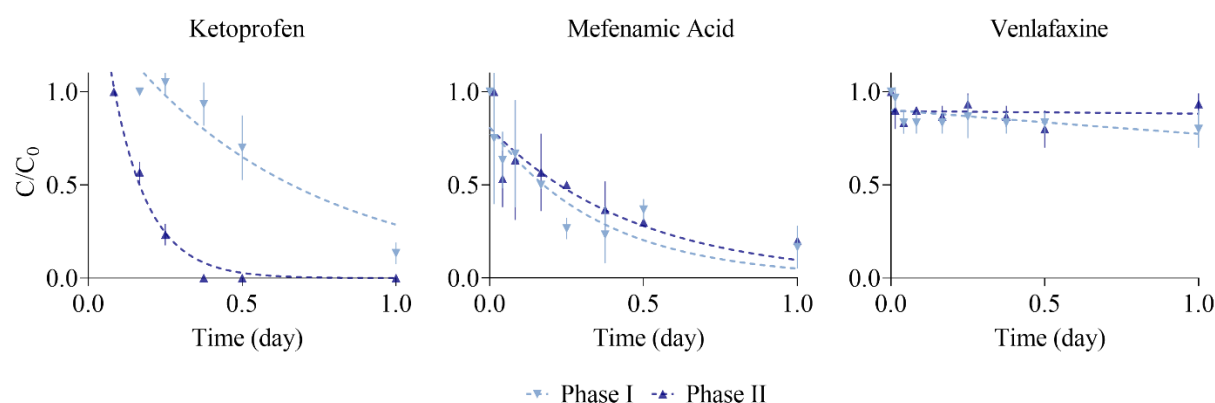


Figure 20: Residual fractions of three representative pharmaceuticals with AGS from Phase I and II (adapted from **Paper V**). The lines represent the first-order degradation kinetics.

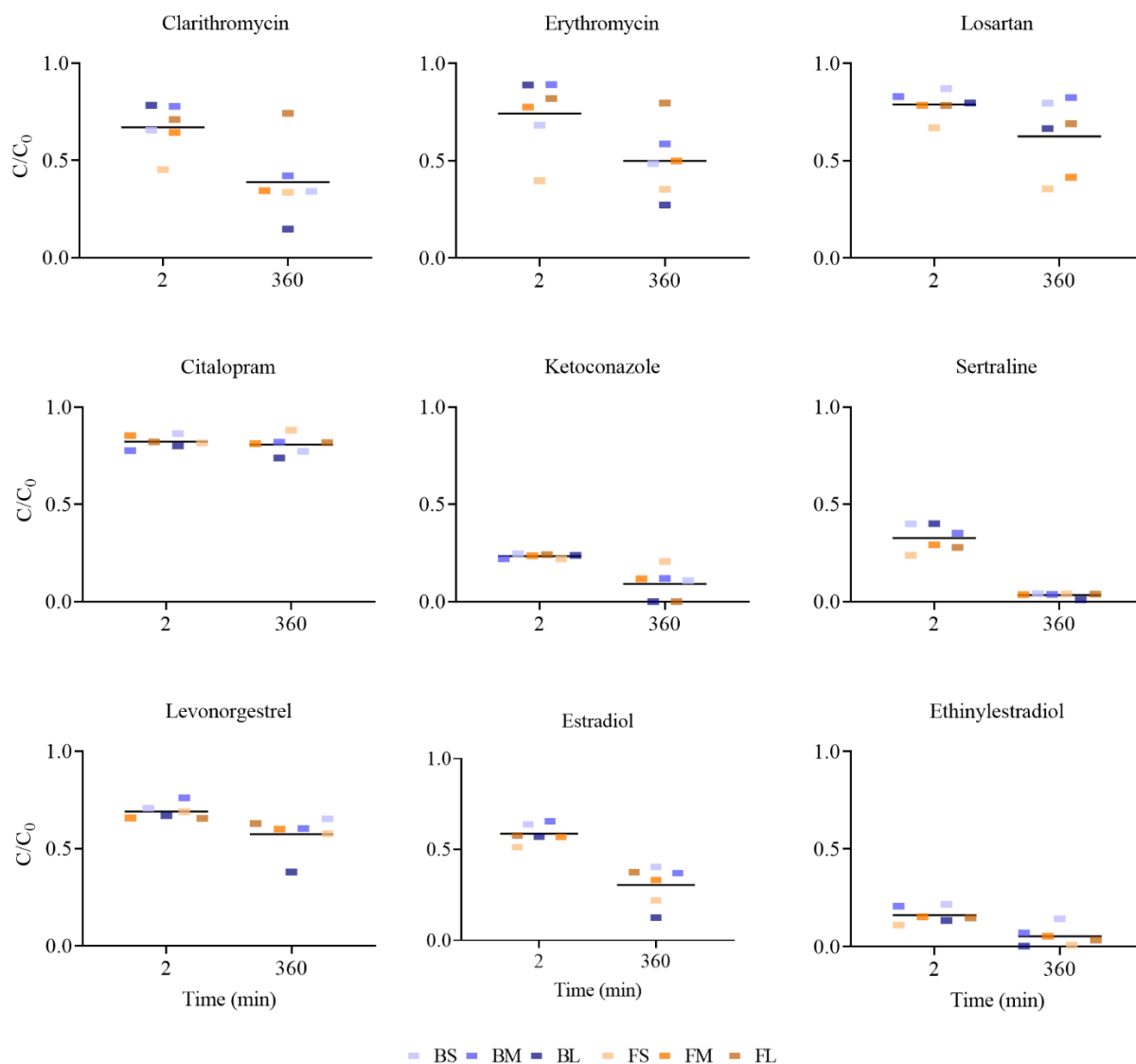
Table 3 summarizes the transformation rate constants obtained in the biological degradation experiments normalized to the biomass concentration. The analysis of the eight compounds revealed that similar transformation rates were observed under the two operational phases. Ketoprofen showed faster removal with AGS biomass adapted to higher DO levels suggesting a positive effect of increased oxygen concentrations on the transformation of this compound. The higher DO might have selected a community more efficient at transforming ketoprofen, and/or could have increased the activity of the microorganisms involved in the transformation process. It cannot be excluded that the increase in removal efficiency may be also attributed to the evolution of a microbial community capable of ketoprofen transformation, independently of the DO concentration in the reactor. Carbamazepine and venlafaxine were recalcitrant to degradation in all batch experiments.

Table 3: Biological transformation rate constants (k_{bio}) normalized to biomass concentration with AGS acclimatized to Phase I and Phase II. The 95% confidence intervals are indicated by $\pm$. Rate constants of $0.07 \text{ L gSS}^{-1} \text{ d}^{-1}$ correspond to the limit of experimental resolution in this study.

Compound	$k_{bio} (\text{L gSS}^{-1} \text{ d}^{-1})$	
	Phase I	Phase II
Atenolol	0.43 ± 0.10	0.35 ± 0.34
Carbamazepine	≤ 0.07	≤ 0.07
Ciprofloxacin	0.31 ± 0.05	0.20 ± 0.06
Citalopram	0.08 ± 0.08	≤ 0.07
Ketoprofen	0.49 ± 0.20	2.65 ± 0.37
Mefenamic Acid	0.83 ± 0.37	0.65 ± 0.43
Sulfamethoxazole	0.28 ± 0.06	0.40 ± 0.07
Venlafaxine	≤ 0.07	≤ 0.07

4.4. Removal by sorption

In **Paper III**, the sorption behavior of 21 selected pharmaceuticals was investigated with two types of lab-scale AGS, one dominated by fungal mycelia and one dominated by bacterial biofilm, at different controlled diameters of 0.5-1, 1-2, and >2 mm. Out of the 21 target compounds, nine pharmaceuticals, namely citalopram, clarithromycin, erythromycin, estradiol, ethinylestradiol, ketoconazole, levonorgestrel, losartan, and sertraline, were removed (> 20% in at least one sample) in the sorption test (Figure 21). Sorption coefficients $K_{d,6h}$ were calculated for the compounds (Table 4) at different biomass conformations. The obtained sorption coefficients were comparable to the values observed in the literature for activated sludge, membrane bioreactor biomass, and moving bed biofilm reactor. Similar observations were made at the full-scale WWTP (**Paper II**) where comparable removal was observed between AGS and CAS for compounds having low residual fractions attributed to sorption mechanisms, like ketoconazole and sertraline. No significant differences were observed between the fungal and bacterial biomass (two-tailed t-test on K_d values, p-value > 0.05), nor with the granule size (two-tailed Welch's t-test on K_d values, p-value > 0.05). This corroborates earlier findings by Lucas et al. (2018), who investigated the sorption of a few pharmaceuticals to fungal biomass and obtained comparable values to activated sludge.



Granule diameter	0.5-1 mm	1-2 mm	>2 mm
Bacteria dominated	BS	BM	BL
Fungi dominated	FS	FM	FL

Figure 21: Concentration of selected pharmaceuticals in the liquid phase at two minutes and six hours in the batch exposed to different biomass conformations (BS, BM, BL, FS, FM, FL). The concentrations are normalized to the initial values. The line describes the mean values (n=6).

The sorption onto the biomass occurred quickly and for most of the compounds, except for citalopram, increased over time (one-tailed Welch's t-test on normalized pharmaceutical liquid concentrations, p -value < 0.05 , Figure 21). It is possible that time enhanced intramolecular diffusion, allowing the pharmaceuticals that quickly adhered to the exterior surface of the granules to diffuse into the interior via the porous structure. Similar behavior was observed in other studies with granules (Alvarino et al., 2015; Shi et al., 2011).

The extent of sorption to the granules varied greatly among the compounds investigated. All the pharmaceuticals that showed removal during the sorption tests are compounds usually detected in the sludge matrix indicating moderate to high affinity to biosolids. The highest sorption coefficients ($K_d > 10^4$, Table 4) were obtained for ketoconazole, sertraline, and ethinylestradiol. The sorption to sludge is considered the principal mechanism responsible for the removal from wastewater of ketoconazole and sertraline (Gornik et al., 2020; Svahn and Björklund, 2019). Ethinylestradiol showed high concentrations of above 200 mg kg⁻¹ MLSS when extracted from sludge samples (Xue et al., 2010).

The nature of the intermolecular interactions between the OMPs and sludge can be hydrophobic and/or electrostatic, thus sorbate and sorbent structural properties are expected to determine which mechanisms of sorption are predominant (Rybacka and Andersson, 2016). The tested pharmaceuticals contain a multitude of functional groups and moieties capable of engaging in several sorption mechanisms. Ionic species sorption is suggested to be mainly promoted by electrostatic attraction to specific surface sites, while electrically neutral compounds could primarily adhere via hydrophobic partitioning and electron donor-acceptor interactions (MacKay and Vasudevan, 2012). Neutral compounds with relatively high lipophilicity, such as estradiol, levonorgestrel, ethinylestradiol, and ketoconazole, demonstrated high mass losses in the water phase which may be attributed to sorption through hydrophobic interactions with the biomass. The partition behavior of losartan, which at pH 7 is mostly present in the negative ionized form, could also be due to its lipophilic behavior ($\text{LogD} = 4.05$). 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). The removal of OMPs present in their cationic form (clarithromycin, erythromycin, sertraline, and citalopram) could be attributed to cation exchange with the negatively charged biomass. As the biomass typically has a negative surface charge, positively ionized micropollutants are likely to show the highest potential for sorption, due to electrostatic attraction (Franco and Trapp, 2008).

Interestingly, no affinity was observed for ciprofloxacin, a zwitterionic fluoroquinolone antibiotic with low hydrophobicity. Ciprofloxacin has been previously reported to partition onto activated sludge (Polesel et al., 2015; Wang et al., 2017) and aerobic granular sludge (Amorim et al., 2014; Ferreira et al., 2016), predominantly via electrostatic interactions (Polesel et al., 2015).

Results and Discussion

Table 4: Sorption coefficients calculated from the concentration measured at 6 hours during the batch experiments for nine pharmaceuticals. Literature K_d values refer to partition coefficients experimentally obtained in secondary sludge, membrane bioreactor biomass, and moving bed biofilm reactor.

	K _{d,6h} (L kg SS ⁻¹)						K _d literature (L kg SS ⁻¹)
	BS	BM	BL	FS	FM	FL	
Hydrophobic compounds LogD > 3.2							
Ketoconazole	2.2·10 ⁴	6.8·10 ³	7.1·10 ⁴	5.8·10 ³	1.1·10 ⁴	1.2·10 ⁵	8.5·10 ³ ^a
Levonorgestrel	1.2·10 ³	5.4·10 ²	1.0·10 ³	1.1·10 ³	9.0·10 ²	6.5·10 ²	2-2.6·10 ² ^a
Losartan	6.3·10 ²	1.8·10 ²	3.2·10 ²	2.7·10 ²	1.9·10 ²	5·10 ²	2.5·10 ² ⁱ
Estradiol	3.6·10 ³	1.4·10 ³	4.3·10 ³	5.3·10 ³	2.7·10 ³	1.8·10 ³	2.3·10 ² ^a , 0.5-0.7·10 ^{3c} , 4.4·10 ⁴ ^d
Ethinylestradiol	1.5·10 ⁴	1.1·10 ⁴	1.8·10 ⁵	1.7·10 ⁵	2.4·10 ⁴	3.0·10 ⁴	1.1-1.5·10 ³ ^c , 6.4·10 ⁵ ^d , 0.9-1.3·10 ³ ^g
Moderately hydrophobic compounds LogD < 3.2							
Clarithromycin	4.6·10 ³	1.1·10 ³	3.6·10 ³	2.9·10 ³	2.5·10 ³	3.8·10 ²	2.6-4.0·10 ² ^b , 0.73- 1.2·10 ³ ^e , 3.4·10 ² - 5.6·10 ³ ^h , 2.5·10 ² ⁱ
Erythromycin	2.5·10 ³	5.8·10 ²	1.6·10 ³	2.7·10 ³	1.3·10 ³	2.8·10 ²	0.7-1.8·10 ² ^f , 2.0·10 ² -6.1·10 ³ ^h
Citalopram	7.3·10 ²	1.9·10 ²	2.2·10 ²	2.1·10 ²	3.2·10 ²	2.5·10 ²	2.1·10 ² ^a , 4.6·10 ² - 2.0·10 ³ ^h , 1.5·10 ³ ⁱ ,
Sertraline	5.9·10 ⁴	2.1·10 ⁴	6.5·10 ⁴	3.6·10 ⁴	3.6·10 ⁴	2.7·10 ⁴	1.7·10 ⁴ ^a , 2.5·10 ⁵ ⁱ
MLSS (g SS L ⁻¹)	0.4	1.2	1.6	0.7	0.7	0.9	
^a Hörsing et al., 2011		^d Xue et al., 2010			^g Banihashemi and Droste 2014		
^b Göbel et al., 2005		^e Abegglen et al., 2009			^h Torresi et al., 2017		
^c Stevens-Garmon et al., 2011		^f Radjenović et al., 2009b			ⁱ Golovko et al. 2021		

4.5. Localization of pharmaceuticals in the biofilm matrix

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging was applied to investigate and analyze the localization of few pharmaceuticals in the complex biological matrix of aerobic granular sludge (**Paper IV**). ToF-SIMS allows displaying the intensity distribution of selected ions on the surface of the biofilm sections. Each ion represents an identified molecule. The intensity of specific ions in each measurement point can be presented in a two-dimensional map, resulting in ion images of the biofilm area analyzed. Positive ion images with a 4x4 mm² area were obtained from the analyzed biofilm (Figure 22). SEM images of the AGS slice revealed that pharmaceutical peaks detected with SIMS correlate with the biofilm surface (Figure 22a and b). Several pharmaceuticals and their localization were identified in the biofilm section, including ciprofloxacin, citalopram, ketoconazole, and sertraline. These compounds are usually detected in the sewage matrix showing moderate to high affinity to biosolids (Hörsing et al., 2011). The distribution of the four identified compounds over the biofilm section is presented in Figure 22c and d.

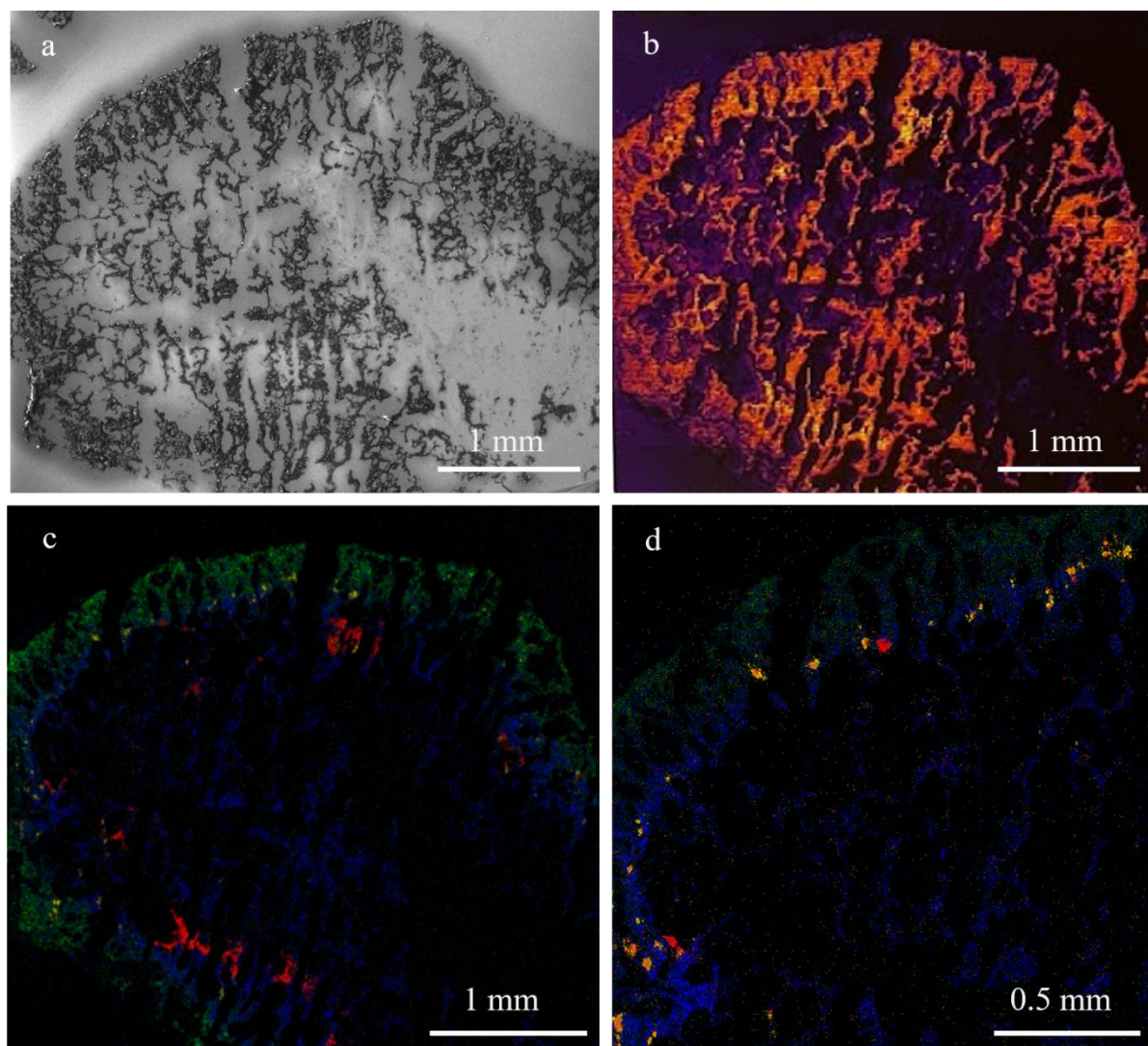


Figure 22: SEM images of the treated biofilm section (a). Total ion image of the treated biofilm section (b). Overlay of ion distributions of selected ion peaks m/z 271.0, 275.0, 325.1, and 495.1, representing respectively ciprofloxacin in red, sertraline in green, citalopram in blue, and ketoconazole in yellow (c, d).

The images revealed heterogeneity in the distribution of different OMPs in the granule. Citalopram penetrated deeply into the structure of the biofilm, whereas sertraline was sequestered in the outer layer of the granule. Ciprofloxacin and ketoconazole accumulated in several hotspots in the biofilm, i.e., relatively small areas with a particularly strong signal. Ketoconazole transformation products were also identified in the biological matrix (refer to **Paper IV**).

The distribution of the identified molecules indicated that different mechanisms played a role in the sorption and transformation of the pharmaceuticals in the biofilm matrix. The buildup of ketoconazole and ciprofloxacin in several hotspots with high intensity, rather than a more homogeneous distribution in the biofilm like for sertraline and citalopram, could indicate the accumulation in the vesicles of microorganisms. Fluoroquinolones such as ciprofloxacin are lysosomotropic substances that can concentrate in lysosomes, where they preferentially localize (Ouédraogo et al., 2007). Amine-containing pharmaceuticals have been also shown to accumulate via ion trapping in the acidic vesicles, such as lysosomes and endosomes, of eukaryotic microorganisms and within the protozoic cells of the activated sludge community (Gulde et al., 2018). Protozoa, such as ciliates, flagellates, and amoebae, are eukaryotes commonly present in wastewater sludge and they can make up over 9% of the volatile solids in activated sludge systems (Madoni, 2010). Protozoa were also frequently observed in the cultivated granular sludge used as biofilm in this investigation (Figure 23).

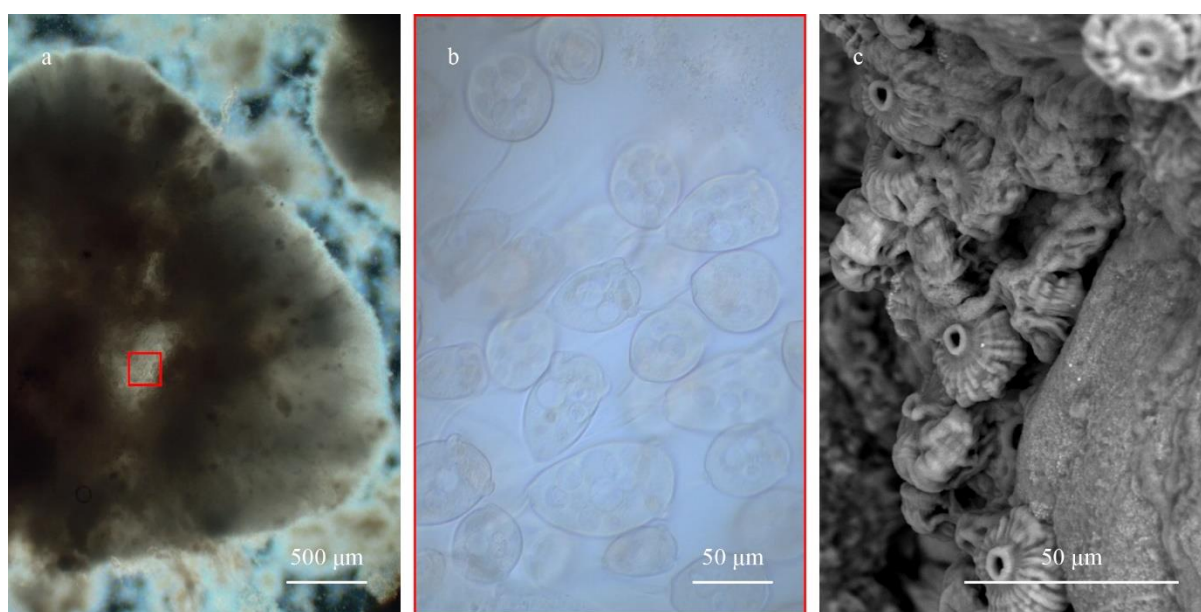


Figure 23: Light microscopy (a, b) and SEM (c) images of the cultivated aerobic granules with visible protozoa on the surface and within the biofilm matrix. The image on the left was taken at 2x magnification (scale bar 500 µm), while the middle image was taken at 20x magnification on the same granule in the highlighted area (scale bar 50 µm).

ToF-SIMS also provided molecular recognition of endogenous biological molecules, enabling the visualization of constituents of EPS and cell components together with the distribution of pharmaceuticals in the biofilm. The signature ions of a cell membrane phospholipid fragment and adenine are shown in Figure 24. Lipids are commonly found in the EPS matrix of biofilms that treat wastewater (Flemming and Wingender, 2010) and they have been found to accumulate at the outer layer of aerobic granules (Chen et al., 2007). Lipids play an important role in the hydrophobic properties of sludge (Conrad et al., 2003) and they can help to emulsify the hydrocarbon substrate and increase its bioavailability (Flemming and Wingender, 2010). Adenine is a nucleic acid marker for bacterial cytoplasm and extracellular DNA, which allows imaging of bacterial distribution within the biofilm (Zhang et al., 2020). The adenine peak had higher intensity in the interior of the granule, possibly related to higher amounts of extracellular DNA.

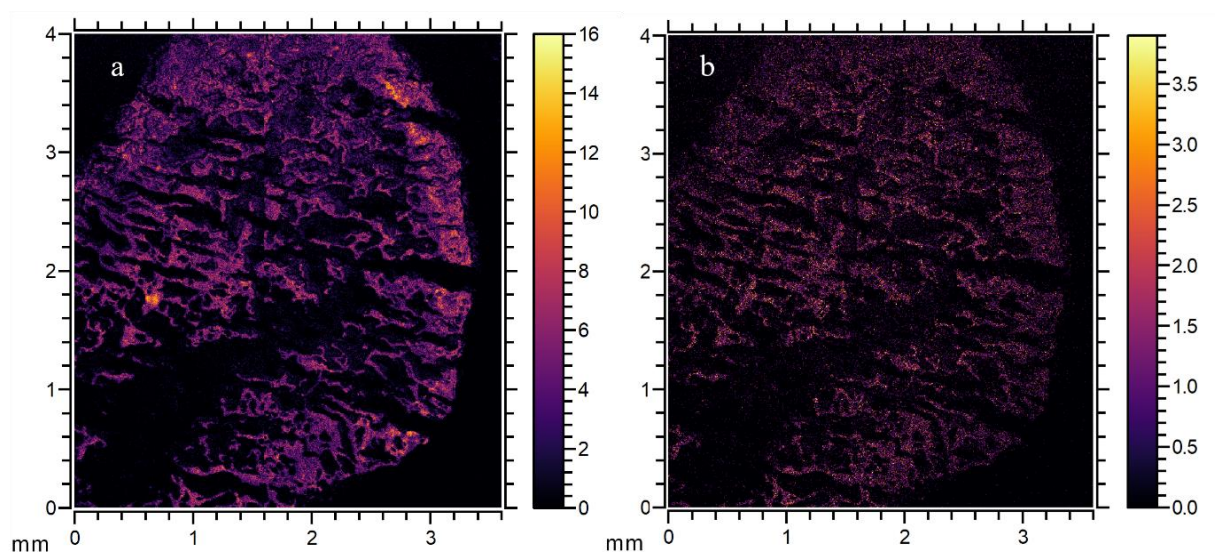


Figure 24: Lipid (a) and adenine (b) distribution in the biofilm slice. The color scale on the right indicates the relative SIMS signal intensity from high (white/yellow) to low (black/purple).

Sertraline and citalopram reflected similar distributions as the lipids and adenine, respectively. Both these antidepressants have been observed to accumulate in several aquatic organism tissues, and mechanisms, other than hydrophobicity, have been suggested to contribute to the distribution of antidepressants in fish brains (Silva et al., 2015). Citalopram and sertraline are present in a cation form at the circumneutral pH, therefore, the sorption could be governed via electrostatic interactions with negatively charged biofilm sites (MacKay and Vasudevan, 2012). Not only do the cell walls of Gram-positive bacteria and the outer membrane in Gram-negative bacteria contain anionic lipid molecules, but also the plasma membranes are negatively charged due to the presence of anionic phospholipids (Malanovic and Lohner, 2016). The colocalization of lipids and antidepressants could suggest that the sorption of citalopram and sertraline might occur through cation exchange of the protonated amine with the anionic sites of the bacterial membrane and cell wall. Citalopram and sertraline also showed complementary distribution in the biofilm slice. Higher intensity of citalopram was observed towards the core of the granule, whereas sertraline concentrated mainly in the periphery. This might suggest a competition for preferred binding sites.

5. Conclusions

The goal of this project was to assess the biological removal of OMPs from wastewater, with a focus on the AGS system.

The experimental results of this work showed that even though biological processes are contributing to the removal of OMPs, their elimination is incomplete and residuals of micropollutants are found in the wastewater effluents. The removal efficiency of OMPs in biological wastewater treatment is dependent on the compound characteristics, the operational conditions, the type of biomass, and the microbial community.

Removal efficiencies by biological treatments vary greatly among the investigated compounds. Some OMPs can be removed efficiently during biological processes from the water phase, i.e., ibuprofen, paracetamol, naproxen, ketoconazole, and bisphenol A. Others, such as carbamazepine, fluconazole, and propranolol, are resistant to microbial degradation by activated sludge and aerobic granular sludge treatments.

At the full-scale WWTP where AGS and CAS are operated in parallel, the highest OMP removal efficiencies were observed in the conventional activated sludge line. A higher transformation capacity of OMPs per unit of biomass was also observed for activated sludge compared to granular sludge in the batch experiments for all but one OMP. The lower OMP removal capacity observed in AGS could be due to the higher mass transfer limitation in biofilm systems. Even though granular biomass showed lower potential for micropollutant transformation, AGS systems had somewhat higher gene cluster diversity compared to activated sludge, which could be related to higher functional diversity. Other factors, such as micropollutant exposure to biomass or mass transfer limitations, therefore played more important roles in the observed differences in OMP removal.

Redox states affect the OMP removal. Oxic conditions supported the microbial degradation of several micropollutants with a faster and/or comparable rate compared to anoxic conditions. Some pharmaceuticals, such as atenolol and sulfamethoxazole, can be transformed both oxidically and anoxically, while other compounds, such as naproxen and zolpidem, appear to be more stable in the absence of molecular oxygen.

Similar pharmaceutical transformation rates were observed under different levels of dissolved oxygen in a lab-scale reactor, except for ketoprofen which increased several folds its removal rate with AGS adapted to higher dissolved oxygen concentration.

Sorption of OMPs to biomass is an important removal mechanism from the water phase. High affinity to AGS was observed for five hydrophobic compounds with different ionization, namely levonorgestrel, estradiol, ethinylestradiol, ketoconazole, and losartan, and four hydrophilic positively charged pharmaceuticals, i.e., sertraline, citalopram, clarithromycin, and erythromycin. Comparable sorption coefficients were obtained for the fungal and bacterial biomass.

Sorption is a relatively fast process. Partitioning of the pharmaceuticals to the biomass was observed already after two minutes from the inoculation. The extent of sorption increased over time for most pharmaceuticals, suggesting that the compounds can penetrate the deeper biofilm matrix. This observation was also confirmed by the chemical analysis of AGS by ToF-SIMS.

Different mechanisms play a role in the sorption and the transformations of the compounds. ToF-SIMS was successfully applied to localize and image pharmaceutical compounds as well as endogenous molecules in the complex biological matrix of granular biofilms for wastewater treatment. Several compounds and their localization were identified in the biofilm section, including ciprofloxacin, citalopram, ketoconazole, ketoconazole transformation products, and sertraline. The results revealed that the pharmaceuticals accumulated in different locations of the AGS structure, indicating that the interactions between the OMPs and the biomass happen at specific sites distributed across the biofilm. Citalopram penetrated deeply into the structure of the biofilm, whereas sertraline was mainly sequestered in the outer layer of the granule. Both compounds seemed to mainly colocalize with phosphocholine lipids. Ciprofloxacin and ketoconazole concentrated in small areas with relatively high intensity. The accumulation in the acidic vesicles of protozoa could be an important mechanism for the sorption and transformation of these antimicrobials.

6. Implications and further research

Research about OMP removal in biofilm systems is necessary to develop strategies to optimize the elimination of such contaminants in the biological processes that already exist in most of the WWTPs, exploit the advantages of biofilm characteristics, and limit the use of resource-intensive physicochemical treatment technologies and harmful side effects. Therefore, it is of particular interest to investigate:

- The differences in removal performances among biofilm systems, such as AGS and MBBR. Higher biotransformation capacity compared to floccular sludge has been observed by biofilm on carriers, while in contrast granular sludge has been observed to transform pharmaceuticals to a lower degree. A possible answer might lie in the density of the biofilm as discussed in section 4.2.2.
- The degree of transformation of micropollutants and the consequential generation of transformation products in AGS. The identification and quantification of transformation products are becoming necessary for an in-depth understanding of the biotransformation processes. Given the abundance of transformation products in the environment, as well as their possible toxic action and synergistic effects, future research should also include transformation products when studying the fate of OMPs and their impact on water quality.
- The microbial community that promotes the biological removal of micropollutants. AGS has been observed to remove OMPs with slower kinetics compared to activated sludge. For bisphenol A, lower concentrations were observed in the effluent of the granular reactors compared to the activated sludge reactors, despite the lower retention time. Could this observation suggest that there are potential microorganisms adapted to the presence of certain recalcitrant compounds and benefit from them through direct metabolism? Meta-omics association studies are a promising approach to understanding and predicting variability in OMP biotransformation efficiency among different microbial communities (Johnson et al., 2015b).
- The transformation of OMPs after sorption to the biomass. Sorption to biosolids results in a longer residence time in the reactor, which may lead to further removal via biotransformation.
- The role in the sorption and biotransformation of micropollutants by microorganisms such as fungi and protozoa which are normally found in wastewater biological treatment and harbor a wide diversity of catabolic capacities.
- The accumulation of OMPs in the foam generated on the top of biological reactors. Enrichment in the foam is a mechanism that might affect several pharmaceuticals and trigger several follow-up questions that are relevant for practical applications. Firstly, how relevant is foam fractionation for organic micropollutants such as pharmaceuticals in full-scale systems? Since aeration is a common feature in wastewater treatment, investigations are needed to quantitatively determine whether pharmaceuticals could be enriched in the foam commonly present on top of biological reactors.

If the magnitude of this mechanism in full-scale systems is environmentally relevant, it may have important implications for sampling approaches when determining pharmaceutical concentrations in wastewater. Secondly, if foam partitioning affects organic micropollutants that are particularly recalcitrant, is it possible to exploit this mechanism for their removal from the water phase? In adsorptive bubble separation processes, the adsorbed compounds are separated by removing the foam mechanically (Buckley et al., 2021). Thirdly, could the enrichment in the foam explain the wide uncertainties in the removal performances at the full-scale wastewater treatment plants of compounds like clarithromycin and erythromycin? Several works have reported poor removal rates, often negative removal performances, with wide uncertainties of these compounds in wastewater treatment processes (Burzio et al., 2022; Golovko et al., 2014; Verlicchi et al., 2013).

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