# Microalgae-based technology for wastewater treatment

## Exploring organic micropollutants removal

Kaiyi Wu

# Propositions

1. Microalgae can efficiently biodegrade persistent organic micropollutants.
   (this thesis)

2. A single microalgal species is not able to remove a wide range of organic micropollutants.
   (this thesis)

3. Visualisation increases scientific impact.

4. Efficient communication in research requires consistent use of terminology.

5. Thinking about avoiding mistakes triggers mistakes.

6. Life is like chocolate, bitter-sweet.

Propositions belonging to the thesis, entitled

Microalgae-based technology for wastewater treatment: Exploring organic micropollutants removal

Kaiyi Wu
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# Microalgae-based technology for wastewater treatment: Exploring organic micropollutants removal

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**Thesis**

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*To my beloved family*

*谨以此书献给我的家人*

# Table of contents

# Chapter 1

# General introduction

# 1.1 Organic micropollutants

Organic micropollutants (OMPs) are a wide group of synthetic organic chemicals, which are discharged into the aquatic ecosystems and present from ng/l to µg/l (Anjum et al., 2017; Vadiraj et al., 2021). All over the world, OMPs, including pharmaceuticals, personal care products and pesticides, have been detected in surface water (Bu et al., 2015; Du et al., 2023), ground water (Sackaria and Elango, 2020; Tisler et al., 2022), drinking water (Ren et al., 2020; Tröger et al., 2020), and wastewater (Clara et al., 2004; Rogowska et al., 2020). Furthermore, the global production of chemicals is rapidly increasing (79% increase from 2000 to 2017) and is predicted to become three times higher in 2050 than 2010 due to the increase of global demand (Persson et al., 2022). If no preventing and mitigating measures are taken, this will result in an ever-increasing emission of OMPs into the environment.

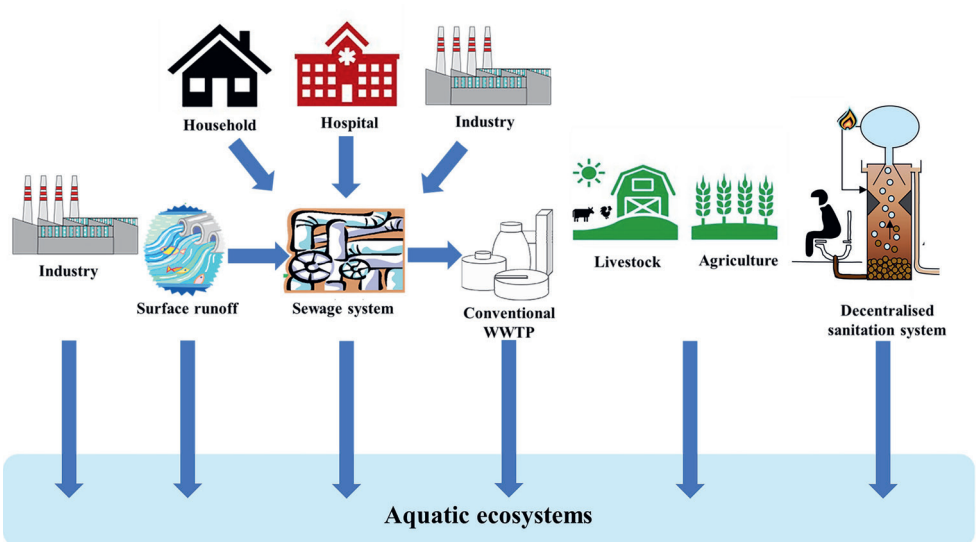



**Figure 1.1** Main sources and transportation pathways of organic micropollutants in the aquatic environment, adapted from Fernandes et al. (2015), Koenis et al. (2015), Kim and Zoh (2016), and Mukhopadhyay et al. (2022).

OMPs enter aquatic ecosystems by multiple point sources and diffuse sources (Figure 1.1). Point sources are the main sources of OMPs and include household, industry and hospital (Ellis, 2006; Kim and Zoh, 2016). Wastewater with OMPs from these sources and part of surface runoff gather in the sewage system and are subsequently treated in conventional WWTP. Since conventional WWTP are not designed for OMPs removal, only a few OMPs are efficiently removed, such as ibuprofen, paracetamol and natural hormones (Margot et al., 2015). Persistent OMPs, such as diclofenac, irbesartan and sotalol, are less than 20% removed, thereby resulting in relatively high concentrations in aquatic ecosystems (Margot et al., 2015; Grandclément et al., 2017). Additionally to WWTP, there are other systems that treat wastewater, e.g. decentralized wastewater sanitation systems that treat black water and grey water (Figure 1.1). For example, black water is treated by anaerobic digestion, and showed poor removal of OMPs, such as diclofenac and carbamazepine (Kujawa-Roeleveld et al., 2008; Moerland et al., 2022). Diffuse and other sources of water with OMPs originate from industry, livestock, agriculture, and surface run-off (Kosek et al., 2020). OMPs from these sources are generally not treated, such as pesticides from agriculture and surface run-off (Mukhopadhyay et al., 2022).

When OMPs enter the aquatic ecosystems, they can negatively affect the metabolism and therefore growth of target and non-target organisms (Sigmund et al., 2023). Exposure to citalopram or methamphetamine at 1 µg/l increased the fish aggression for 42 days (Hubená et al., 2021). Tetracycline at 10 to 100 µg/l led to an irreversible decline in the populations of periphyton (nematode, bacteria and algae) in mesocosm stream for 28 days (Quinlan et al., 2011). Additionally, 34 to 95% decline of vulture populations was observed between 2000 and 2003 in India and Pakistan, due to the presence of diclofenac in their food (Oaks et al., 2004). To improve ecological water quality, it is crucial to remove persistent OMPs in WWTP and decrease OMPs release through diffuse sources.

## 1.2 Microalgae-based technologies for OMPs removal

Conventional WWTP needs to be upgraded because of the high energy consumption, emission of $CO_2$, and low removal efficiency of OMPs (Guven et al., 2023). Microalgae-based technology is a sustainable alternative for wastewater treatment (Abinandan and Shanthakumar, 2015; Oviedo et al., 2022). Microalgae can efficiently assimilate $CO_2$ and macro nutrients, such as nitrogen and phosphorus, and microelements, such as iron and zinc (Brasil et al., 2017; Suleiman et al., 2020; Xiong et al., 2021; Fernandes et al., 2022). The generated biomass can be harvested by flocculation and sedimentation, and further used for a variety of products, e.g. organic fertilizers and value-added products, including bulk chemicals (Deconinck et al., 2018; Udayan et al., 2022). Furthermore, previous studies have shown that this technology can efficiently remove a variety of OMPs by biodegradation, bioadsorption, bioaccumulation, and photodegradation (Figure 1.2).

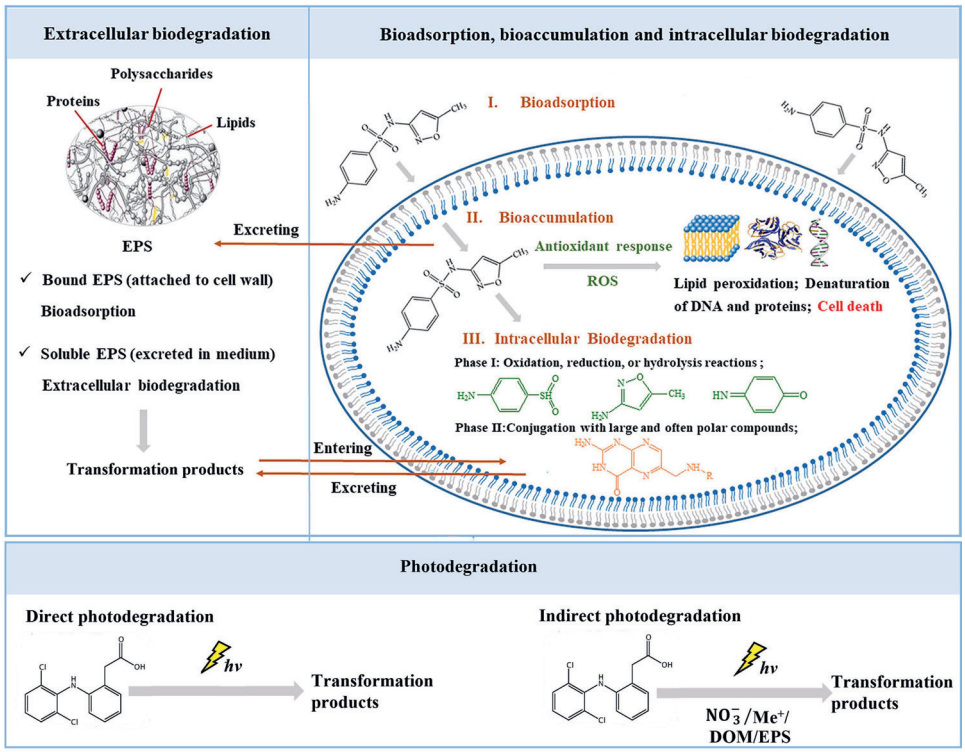



**Figure 1.2** Microalgae-mediated removal processes and mechanisms of OMPs, adapted from Xiong et al. (2021).

## 1.2.1 Biodegradation

Biodegradation is a complex enzyme-mediated process, that can occur intra- and extracellularly (Figure 1.2). In general, the intracellular process (Figure 1.2 - III) is divided into Phase I and Phase II, according to the functionalities of enzymes. In Phase I, the parent compound is broken down through oxidation, reduction, and hydrolysis reactions (Ding et al., 2017; Wang et al., 2023). These reactions are initiated by enzymes, such as cytochrome P450 cytochrome b5 and P450 reductase (Torres et al., 2008; Chu et al., 2022). In Phase II, the conjugations between metabolites and glutathione might occur in the presence of glutathione-S-

transferases, resulting in the opening of epoxide ring to avoid oxidative damage (Ding et al., 2017). Biodegradation can also be mediated by peroxidase (POX) (Vo et al., 2020; Zhao et al., 2021; Li et al., 2022). POX is generated together with reactive oxidative species (ROS), when microalgal cells are stressed by the accumulated OMPs. ROS include hydroxyl radicals and hydrogen peroxidase. POX can oxidize OMPs in the presence of hydrogen peroxidase, e.g. bisphenol A and ibuprofen (Chauhan and Sahoo, 1999; Ugya et al., 2020; Vo et al., 2020). Additionally, enzymes can be excreted to medium, together with soluble extracellular polymeric substance (EPS) that are produced by microalgae. This results in extracellular biodegradation of OMPs (Gao and Chi, 2015; Usmani et al., 2020; Bahman et al., 2022). So far, extracellular biodegradation is seldom studied, but is getting more and more attention (Gao and Chi, 2015; Shi et al., 2018; Wang et al., 2022). As the molecular structures of OMPs are diverse (Sutherland and Ralph, 2019; Liu et al., 2021), biodegradation processes are complex and need to be further explored.

Biodegradation is OMPs type dependent and is generally the predominant removal process for OMPs with strong electron-donating groups (e.g. -$NH_2$ and -OH), such as ciprofloxacin and sulfamethoxazole (Bai and Acharya, 2016; Liu et al., 2011; Xie et al., 2020; Kitamura et al., 2023). Ciprofloxacin biodegradation was higher than 65% in previous studies of batch experiments (Xie et al., 2020; Kitamura et al., 2023). In comparison, biodegradation contributed less or even poorly to the removal of compounds with electron-withdrawing groups (e.g. amide, halogens and cyano group), such as carbamazepine and carbendazim (Zhou et al., 2014; Liu et al., 2021).

Biodegradation is also species dependent, as different species have different biodegradation capacity of OMPs (Hena et al., 2021). *Chlorella pyrenoidosa* showed 40% higher biodegradation of norgestrel than *Scenedesmus obliquus* for 5 days under similar experimental conditions (light, temperature, and air/$CO_2$ flow rate) in batch experiments (Peng et al., 2014). *Scenedesmus obliquus* showed 25% higher biodegradation of climbazole than *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Chlorella vulgaris* for 7 days under similar experimental conditions (light, temperature, and air/$CO_2$ flow rate) in batch experiments (Peng et al., 2014).

The varying biodegradation capacity of different species is attributed to their various growth rate, lipid content, and enzymatic activities (Lei et al., 2003, 2007).

### 1.2.2 Bioadsorption and bioaccumulation

Biadsorption and bioaccumulation occur consecutively, prior to intracellular biodegradation, when microalgae are exposed to OMPs as illustrated in Figure 1.2, by I, II, and III. Bioadsorption is a metabolically passive process where OMPs are adsorbed onto the surface of microalgal cells, or the bound EPS, excreted by microalgal cells (Sutherland and Ralph, 2019; Xiong et al., 2021). Bound EPS is the EPS that is loosely or tightly bounded to the microalgal cells (Matsumoto et al., 2014; Huang et al., 2021). Bioaccumulation is a metabolically active process where adsorbed OMPs accumulate intracellularly. Bioaccumulated OMPs can stimulate the overproduction of antioxidants, such as POX and ROS (Gauthier et al., 2020; Ugya et al., 2020). Although these antioxidants can participate in the biodegradation of OMPs as described in 1.2.1, they can damage the lipid, DNA, and protein, even resulting in cell death (Li et al., 2006; Wan et al., 2015). On the other hand, the presence of bioadsorption and bioaccumulation might increase the risk of biomass for further application. The adsorbed and accumulated OMPs might enter the aquatic and terrestrial ecosystems through biomass-made products, such as fertilizer. Minimizing the contribution of these two removal processes is essential for producing high-quality biomass and avoiding the secondary contamination of OMPs.

### 1.2.3 Photodegradation

Illumination is an essential element for microalgae-based technology and can induce direct and indirect photodegradation of OMPs (Xiong et al., 2018; Liu et al., 2021). Direct photodegradation occurs when OMPs absorb radiation from light with specific wavelength, that results in an excited state of the molecule (Muszyński et al., 2020). When photosensitizers, such as nitrate, DOM, and EPS, are present in the medium, indirect photodegradation occurs in OMPs, like ibuprofen and triclosan, resulting in a higher removal than direct photodegradation (Figure 1.3). Nitrate can adsorb light with < 350 nm wavelength and produce hydroxyl radicals to oxidase OMPs (He et al., 2016; Bavumiragira et al., 2022). Transition metal ion, like $Fe^{3+}$,

form the photosensitive complex with OMPs, such as clarithromycin and roxithromycin, therefore resulting in an efficient photodegradation (Vione et al., 2009; Fatta-Kassinos et al., 2011). On the other hand, DOM and EPS in microalgae-based systems can also mediate OMPs photodegradation (Figure 1.3c, d). DOM with aromatic groups in wastewater can act as photosensitizer by forming active $^3$DOM* to enhance the degradation of OMPs (Huang et al., 2022; Sardana et al., 2022). The $^3$DOM* reactive species are organic moieties in the DOM excited to a higher energy status (Huang et al., 2022; Sardana et al., 2022). They can interact and initiate decomposition of specific electron-rich groups in OMPs, such as phenolic groups (He et al., 2016; Guo et al., 2023). EPS from microalgae can also form light excited triplet states to induce a degradation of OMPs (Tian et al., 2019). EPS from *Chlorella vulgaris*, consisting of protein-like substances, were found to be more likely components involved in indirect photodegradation of oxytetracycline than DOM of Suwannee River (Wang et al., 2022). The role of EPS and DOM in indirect photodegradation of OMPs is not well understood and needs to be further investigated in microalgae-based systems.

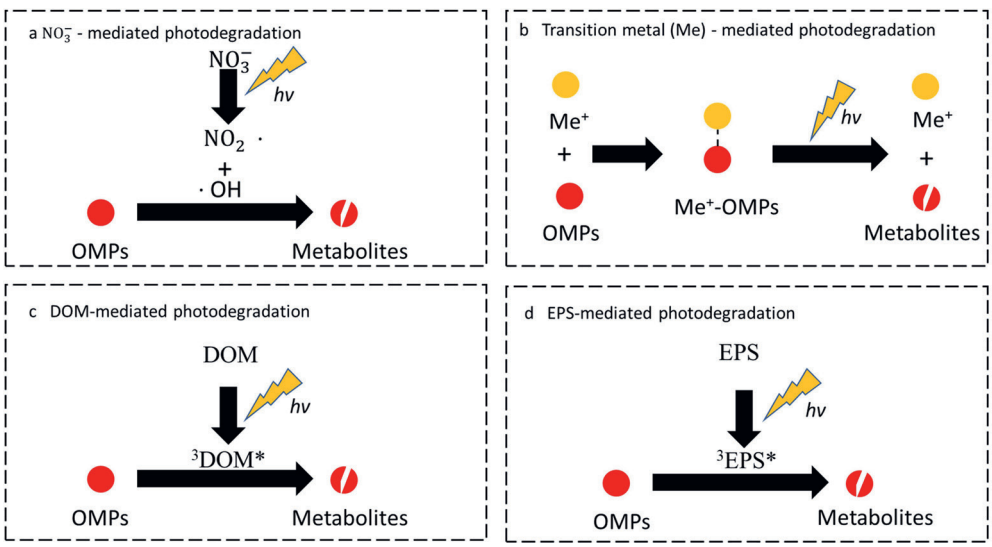



**Figure 1.3** General mechanisms of indirect photodegradation of OMPs.

Photodegradation has proven to be a predominant removal process in microalgae-based systems for photosensitive OMPs, such as diclofenac, tetracycline, and sulfonamides (Garcia-Rodríguez et al., 2013; Sánchez-Sandoval et al., 2022). For example, tetracycline was completely removed by photodegradation for 20 days in batch experiments with *Spyrogira sp.* and *Zannichellia palustris* (Garcia-Rodríguez et al., 2013). This shows that photodegradation can play an essential role for removing OMPs that are hardly biodegradable, but photodegradable.

### 1.2.4 Wastewater characteristics

Wastewater characteristics, like inorganic nitrogen and phosphorus, and soluble COD, can affect the removal of OMPs by influencing the growth of microalgae, or interacting with OMPs.

Basically, when microalgal species are cultivated under repleted nutrient conditions, and of course under sufficient light, they will grow exponentially resulting in higher biomass. This biomass can be measured as dry weight or cell number. As the weight of individual cells varies according to the species, this means that the number of cells might differ from the dry weight in a microalgal community. The increased number of algal cells leads to more enzymes available for the biodegradation of OMPs and more binding sites for the bioadsorption of OMPs, thereby higher removal of OMPs. This apparently is not the only factor, as the removal capacities of OMPs depends on other microalgal physiological aspects, that are dependent on biotic and abiotic interactions, as will be addressed later in this thesis.

The chemical composition of the wastewater can affect the photodegradation of OMPs. For example, nitrate can act as photosensitizer and promote the indirect photodegradation of certain OMPs, like triclosan and atenolol (Ji et al., 2012; Bai and Acharya, 2019). Soluble organic compounds from a natural or anthropogenic origin (often denoted as COD) can have aromatic groups that promote the photodegradation of OMPs, such as amoxicillin, and atenolol, by acting as photosensitizers (Cristale et al., 2017; Sardana et al., 2022). This effect depends on COD concentrations and composition.

The biodegradation of OMPs can also be affected by soluble COD. Soluble COD can bind with OMPs molecules reducing the availability of OMPs to microalgal cells and therefore its biodegradability (Ding et al., 2019; Tong et al., 2020). Overall, the effect of wastewater characteristics on the removal of OMPs is complex.



### 1.2.5 Species richness

Species richness is defined as the number of species in an ecosystem (Colwell, 2009), and is the simplest parameter for quantifying biodiversity (Brown et al., 2007). Increasing biodiversity, e.g. increasing microalgal and bacterial species richness, can improve the functions of microalgae-based systems, such as biomass production and nutrient recovery by interspecies communications through metabolites (Power and Cardinale, 2009; Padmaperuma et al., 2018). For the removal of OMPs, only a few studies demonstrated the positive effect of microalgal species diversity in batch systems (Stravs et al., 2017, 2019; Prosenc et al., 2021; Xiao et al., 2021). These positive effects can be attributed to the more diverse enzymes produced in the algal community with higher species richness. These enzymes can improve the removal by complementing metabolisms for OMPs (Stravs et al., 2017, 2019). On the other hand, adding heterotrophic bacteria to the algal community can also increase the removal of OMPs (Liang et al., 2013; Luo et al., 2014; Xiao et al., 2021). Heterotrophic bacteria can stimulate the growth of microalgae by complementarity in nutrient requirement (Oviedo et al., 2022; Wang et al., 2022). Hence, more enzymes and binding sites are provided for the removal of OMPs (Liang et al., 2013). Also, heterotrophic bacteria can degrade OMPs that are poorly removed by certain microalgal species, like ketoprofen and norfloxacin (Ismail et al., 2016; Xiao et al., 2021). Constructing a highly diverse microalgal-bacterial community apparently can benefit for removing a variety of OMPs (Hena et al., 2021).

Species richness may not always positively affect the removal of OMPs. A mixed community of *Scenedesmus obliquus*, *Chlamydomonas mexicana*, *Chlorella vulgaris*, *Ourococcus multisporus*, and *Micractinium resseri* showed similar removal of enrofloxacin with *Chlorella vulgaris*, which had higher removal than other species (Xiong et al., 2017). A mixed community from high rate algal pond (HRAP) had lower removal kinetic constants of thiacloprid, estrone, and metoprolol

than *Chlorella vulgaris* (Prosenc et al., 2021). This shows that microalgal interspecies and microalgal-bacterial interactions on the removal of OMPs are complex.

## 1.3 Knowledge gaps

So far, the number of studies of microalgae-based technologies for the removal of various OMPs in different types of wastewater or media is not abundant, but slowly increasing (Usmani et al., 2020; Hena et al., 2021; Ali et al., 2023). Also, more species of green microalgae, cyanobacteria, and diatoms are tested in these studies (Sutherland and Ralph, 2019; Ahmad, 2022; Ali et al., 2023). Nevertheless, multiple knowledge gaps need to be addressed for further understanding and applying microalgae-based technology for the removal of OMPs.

The removal processes of OMPs in microalgae-based systems are to a limited extend researched (Matamoros et al., 2016; Kiki et al., 2020; Pan et al., 2021; Chu et al., 2022). These studies focused on systems with either individual compound, or a mixture of OMPs. An insight into the mutual interference of mixed compounds on their removal processes is still largely absent. This also includes the enzymes responsible for the biodegradation of OMPs. Especially the role of peroxidase (POX) in the extracellular and intracellular biodegradation of OMPs needs to be elucidated.

Second, the effect of wastewater characteristics on the removal of OMPs is not clear. Most studies only focused on the removal of OMPs in one type of wastewater or nutrient-sufficient media (Matamoros and Rodríguez, 2016; Hom-Diaz et al., 2017; Škufca et al., 2021; Xiong et al., 2021). Batch biomass incubation in the presence of spiked OMPs was the main method applied in these studies. In other studies, continuous, or semi-continuous mode was applied in investigating the removal of OMPs during biological treatment of WWTP (Singhal and Perez-Garcia, 2016; Grandclément et al., 2017). Investigating the predominant wastewater characteristics under continuous mode can give an insight in the further optimization of microalgae-based wastewater treatment for OMPs removal.

Finally, the knowledge gap of species richness effect on the removal of OMPs needs to be filled. Most studies focused on either individual species, or a mixed microalgal-bacterial community from nature or wastewater treatment facilities (Lei et al., 2007; Hom-Diaz et al., 2017; Liu et al., 2021; Prosenc et al., 2021). The effects of increasing microalgal and bacterial species richness need to be investigated on the removal of OMPs. The studies of both batch and continuous modes can aid in elucidating the contributions of different microbial species to the removal of OMPs from (waste)water.

## 1.4 Thesis outline

This thesis explores the potential of microalgae-based technologies for removal of 16 OMPs from wastewater. The OMPs removal processes, effects of wastewater characteristics and species richness on the removal of OMPs were investigated.

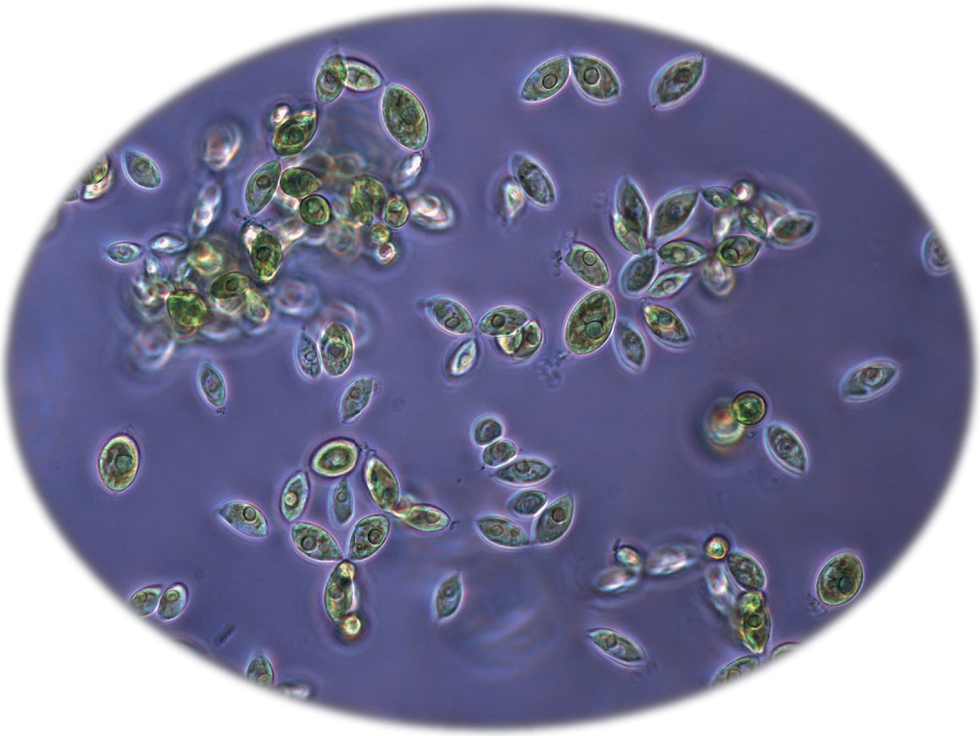
In **Chapter 2**, the contributions of removal processes on the removal of 6 OMPs were assessed when present as individual compounds and in mixture under controlled optimal growth conditions for *Scenedesmus obliquus*. These experiments were conducted in a batch system under fluorescent light. Furthermore, the role of intracellular and extracellular peroxidase on the biodegradation of these OMPs was investigated.

In **Chapter 3**, the impact of wastewater characteristics on the removal of 16 OMPs was assessed by cultivating *Chlorella sorokiniana* in anaerobically digested black water (AnBW), municipal wastewater (MW), and secondary clarified effluent (SCE) under LED light. The experiments were performed in batch mode and the steady state of continuous mode (HRT: 0.8 days). Further, statistical analysis was conducted to elucidate the correlations between wastewater characteristics, biomass, and OMPs removal during steady state.

The effect of microalgal and bacterial species richness on OMPs removal was investigated in **Chapter 4 and 5**. The continuous experiments (HRT: 2 days) of **Chapter 4** were conducted in two 27.5L vertical tubular photobioreactors with 16

OMPs for 112 days under Dutch natural light conditions of spring/summer. One reactor was inoculated with *Chlorella sorokiniana*, another one was inoculated with five green algal species, one cyanobacteria and heterotrophic bacteria from a municipal WWTP. **Chapter 5** included flask experiments and chemostat experiments. Flask experiments with 9 target OMPs were conducted in batch mode under florescent light. Green algae, cyanobacteria and heterotrophic bacteria were involved. Chemostat experiments with 6 target OMPs were conducted in continuous mode (HRT: 5 days) under LED light. Green algae, cyanobacteria, heterotrophic bacteria, and diatoms were inoculated.

In **Chapter 6**, all the findings of the experimental chapters are discussed in relation to OMPs removal processes, effects of wastewater characteristics, operational parameters, and residence time. Based on these findings, research opportunities were identified.



# Chapter 2

# Removal processes of individual and a mixture of organic micropollutants in the presence of *Scenedesmus obliquus*

Kaiyi Wu, Rosaria Tizzani, Hans Zweers, Huub Rijnaarts, Alette Langenhoff, Tânia V. Fernandes

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# Abstract

Organic micropollutants (OMPs) need to be removed from wastewater as they can negatively affect aquatic organisms. It has been demonstrated that microalgae-based technologies are efficient in removing OMPs from wastewater. In this study, the removal processes and kinetics of six persistent OMPs (diclofenac, clarithromycin, benzotriazole, metoprolol, carbamazepine and mecoprop) were studied during cultivation of *Scenedesmus obliquus* in batch mode. These OMPs were added as individual compounds and in a mixture. The short experiments (8 days) were designed to avoid masking of OMPs removal processes by light and nutrient limitation. The results show that diclofenac, clarithromycin, and benzotriazole were mainly removed by photodegradation (diclofenac), biodegradation (benzotriazole), or a combination of the two processes (clarithromycin). Peroxidase was involved in intracellular and extracellular biodegradation when benzotriazole was present as individual compound. Carbamazepine, metoprolol and mecoprop showed no significant biodegradation or photodegradation, and neglectable removal (<5%) by bioadsorption and bioaccumulation. The mixture of OMPs had an adverse effect on the photodegradation of clarithromycin and diclofenac, with reduced first-order kinetic constants compared to the individual compounds. Benzotriazole biodegradation was inhibited by the presence of the mixture of OMPs. This indicates that the presence of the OMPs inhibits the photodegradation and biodegradation of some individual OMPs. These results will improve our understanding on the removal processes of individual and mixtures of OMPs by microalgae-based technologies for wastewater treatment.

**Keywords:** microalgae, OMPs, photodegradation kinetics, biodegradation kinetics, bioaccumulation

# 2.1 Introduction



Organic micropollutants (OMPs), such as pharmaceuticals, personal care products, surfactants, pesticides and herbicides, are present in wastewater at low concentration (ng/l - µg/l) (Nguyen et al., 2020). They can interfere with the endocrine and other bioregulatory systems of aquatic organisms, such as fish and daphnia (Schwarzenbach, 2006; Kristofco et al., 2015). OMPs cannot be efficiently removed by conventional wastewater treatment plants (WWTP) (Sutherland and Ralph, 2019). Thus, many technologies, such as ozonation and activated carbon, have been developed in the past decade to eliminate OMPs from wastewater (Völker et al., 2019; Fundneider et al., 2021).

Microalgae wastewater treatment is a new technology that combines water cleaning with production of microalgal biomass that can be further processed to produce fertilizers, biostimulants, bioplastics and high value products (Renuka et al., 2021). Microalgae-based technologies enable multiple removal processes of OMPs, including photodegradation, biodegradation, bioadsorption and bioaccumulation (Xiong et al., 2018; Usmani et al., 2020). Of these processes, photodegradation can achieve an efficient removal of 40 to 100% of light sensitive compounds, such as ibuprofen (De Wilt et al., 2016). Bioadsorption refers to the adsorption of OMPs on microalgal cell surfaces or extracellular organic substances excreted by the microalgal cells, while bioaccumulation refers to the uptake of OMPs into the microalgal cells (Xiong et al., 2018; Sutherland and Ralph, 2019). Biodegradation is one of the most promising processes since it transforms parent compounds into less toxic molecules (Nguyen et al., 2020; Usmani et al., 2020). P450 enzymes play an important role in the biodegradation of OMPs, as they transform OMPs to smaller molecules with a more hydrophilic nature by adding or unmasking hydroxyl functional groups (Liu et al., 2021). In addition, reactive oxygen species (ROS), such as hydrogen peroxidase and hydroxyl radicals, and antioxidant enzymes like peroxidase (POX), can be produced by microalgae when exposed to OMPs (Xiong et al., 2018; Vo et al., 2020). POX was reported to remove OMPs such as bisphenol A and diclofenac by using hydrogen peroxides as a co-substrate (Chauhan and Sahoo, 1999; Maryskova et al., 2021). Additionally, hydrogen peroxidase can be

converted to highly reactive hydroxyl radicals in the presence of extracellular or intracellular $Fe^{2+}$ via Fenton reaction (Zhang et al., 2019).

Most studies on microalgae-based technologies for wastewater treatment report only the removal efficiency of OMPs and do not study the underlying processes (Maryjoseph and Ketheesan, 2020; Hena et al., 2021). Moreover, the few studies that evaluate these aspects, focus on individual compounds (Escapa et al., 2016; Da Silva Rodrigues et al., 2020; Xiong et al., 2020), and neglect the effects of a mixture of compounds. However, OMPs are often present in mixtures in wastewater (Nguyen, 2021). These co-existing compounds can either promote or inhibit the removal of target OMPs. For example, the presence of sulfamethoxazole (0.5 mg/l) enhanced the removal of sulfamethazine by *Scenedesmus obliquus* from 22 to 53%. Sulfamethoxazole induced the production of the enzymes, aminopyrine N-demethylase and aniline hydroxylase, both taking part in the bioconversion of sulfamethazine (Xiong et al., 2019). The presence of ibuprofen and naproxen enhanced the removal of carbamazepine and sulfamethoxazole, but inhibited the removal of atenolol by the diatom, *Navicula sp.* (Ding et al., 2020). Thus, it is important to study the removal processes and kinetics of OMPs as individual compounds and in mixtures.

In this study, the removal of six individual OMPs and in a mixture of these OMPs was studied during cultivation of *Scenedesmus obliquus* in batch mode. The processes responsible for the removal of these OMPs were identified, and the effect of the mixture of OMPs on the individual OMP removal processes and kinetics were examined. Finally, the role of extracellular and intracellular POX on the biodegradation of OMPs was studied.

# 2.2 Materials and methods

## 2.2.1 Target OMPs

Diclofenac (DCF), clarithromycin (CLA), benzotriazole (BTZ), metoprolol (MET), carbamazepine (CBZ) and mecoprop (MCPP) were selected as target compounds based on their persistence in wastewater treatment and aquatic ecosystems, diversity of therapeutical class, measurability and presence in European wastewater (Giannakis et al., 2015). The concentrations of OMPs were chosen based on studies with similar experimental conditions, set-up and microalgal species. The spiked concentrations of MCPP, DCF and CBZ were 1000 µg/l, as used by Escapa et al. (2016) and Xiong et al. (2016), while the spiked concentrations of BTZ and MET were 300 µg/l, as used by the studies of De Wilt et al. (2016) and Gatidou et al. (2019).



As CLA was not reported in literature when cultivating *Scenedesmus obliquus,* other green microalgae belonging to the same taxa, *S. quadriculata*, *C. vulgaris* and *R. subcapitata* (Guo et al., 2020a; Kiki et al., 2020), were used for reference. In the reported study, the green microalgae species were inhibited by CLA at concentrations higher than 100 µg/l. To avoid the possible inhibition of CLA on the growth of *Scenedesmus obliquus* and ensure sufficient CLA concentration for quantifying all different removal processes, 60 µg/l was determined as the spiked concentration.

To avoid the effect of methanol on the experiments, the OMPs stock solutions in methanol were evaporated by gentle nitrogen gas till dryness, and afterwards the sterilised BG-11 medium was added to get the spiked BG-11 medium.

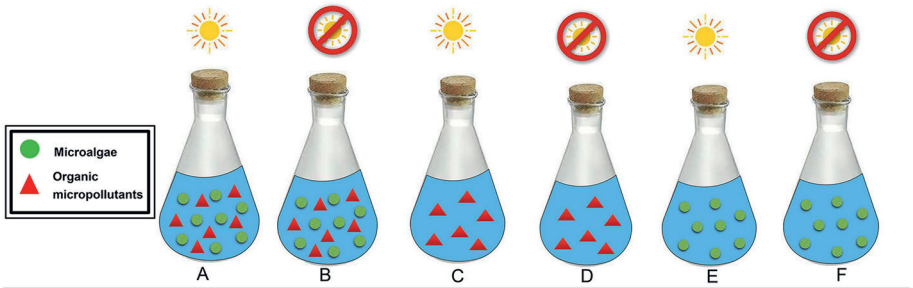
## 2.2.2 Microalgae adaptation

*Scenedesmus obliquus* (CCAP276/3a) originated from the microalgae culture collection of the Netherlands Institute of Ecology (NIOO-KNAW), The Netherlands. It was maintained in WC medium (Kilham et al., 1998) at 35 °C with a continuous irradiation of 80 µmol $m^{-2}$ $s^{-1}$.

*S. obliquus* was first adapted to sterilized BG-11 medium (Table S2.1) until constant growth rate was reported for a minimum of 7 generations. The adaptation was conducted in 300 ml sterilized Erlenmyer flasks closed with cotton-wool stoppers. The flasks were filled with 200 ml sterilized BG-11 medium and inoculated with *S. obliquus* at a chlorophyll a of 6.0 ± 0.8 µg/l. They were placed in a Multitron 2 Incubation Shaker (Infors AG, Switzerland) at 150 rpm at 25 °C, and randomized daily to ensure equal contribution of illumination. The average light intensity was 126 µmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 12/12 h). Air enriched with 10% $CO_2$ was used to aerate the incubator at a flow rate of 120 l/h.

### 2.2.3 Experimental set-up

The experiments with six individual compounds or the mixture were performed in 300 ml flasks as described at 2.2.2. The experiments lasted 8 days to prevent light and nutrient limitation. Longer experimental period would result in microalgae self-shading and therefore light limitation within the flasks. The possible microalgal growth limitations could interfere with the removal processes of OMPs as metabolic pathways might change in depleted conditions. All experiments were performed in triplicate. To investigate the removal processes of OMPs, two treatments were applied: A) microalgae cultivation spiked with OMPs exposed to light, and B) microalgae cultivation spiked with OMPs in the dark (flasks wraped with aluminum foil). In order to quantify the OMPs removal without microalgae, two abiotic controls were performed: C) OMPs exposed to light and D) OMPs in the dark. Finally, to investigate the effect of OMPs on microalgal growth, two biotic controls were performed: E) microalgae cultivation exposed to light and F) microalgae cultivation in the dark. Both controls performed in the dark (D and F) were anticipated to remain unchanged during the experiment, as no decomposition of the OMPs (D) or microalgae growth in the dark (F) was expected. They were performed to experimentally confirm the expectations.




| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Photodegradation | + | − | + | − | − | − |
| Biodegradation | + | + | − | − | − | − |
| Bioadsorption | + | − | − | − | − | − |
| Bioaccumulation | + | − | − | − | − | − |

**Figure 2.1** Schematic representation of the batch experiments with conditions supportive (+) or non-supportive (-) to potential removal processes for each treatment and control.

OMPs removal in control C was denoted as photodegradation. Bioadsorption and bioaccumulation were determined as described in 2.2.4. The difference between the removal of OMPs in treatment A and photodegradation, bioadsorption and bioaccumulation was denoted as biodegradation.

## 2.2.4 OMPs extraction

Bioadsorbed and bioaccumulated OMPs were extracted according to the work-flow procedure in Figure S2.1. A volume of 50 ml of microalgal sample was taken in duplicate from treatment A at the end of the experiment and centrifuged (4500 rpm, 4 min). The pellet was washed (Three times) with 50 ml of milli-Q water. The OMPs released from pellet to the washing milli-Q water were the bioadsorbed OMPs (Xiong et al. 2016; Kiki et al. 2020; Vo et al. 2020). These OMPs were extracted by solid phase extraction (SPE). The washing milli-Q water and internal standards (Table S2.2) were loaded on the SPE cartridges (Oasis HLB 6cc, 200 mg, Waters Corporation, Hilford, USA), which were pre-conditioned with 5 ml of methanol and

pre-equilibrated with 5 ml of milli-Q water. Afterwards, the absorbed impurities on the cartridges were washed out by 5 ml of milli-Q water. When no further drops were observed, 10 ml of methanol was added to elute the loaded OMPs. The methanol was dried under a gentle nitrogen flow and redissolved in 1 ml of acetonitrile/milli-Q water (v/v, 7/3). After centrifugation (4500 rpm, 10 min), the supernatant was collected and injected into a liquid chromatograph coupled to a triple quadruple mass spectrometer (LC-MSMS) with an electrospray ionization source. The SPE recoveries of target compounds, determined as the ratio of measured amount to added amount of their internal standards, were 45 to 125% with a standard deviation up to 12% (Table S2.2). Bioadsorption was evaluated by the ratio between the amount of bioadsorbed OMPs and the initial amount of OMPs.

The OMPs that remained in the pellet, the bioaccumulated OMPs. These OMPs were extracted by Quechers standard method (Lehotay, 2007). For this, the pellet and internal standards (Table S2.2) were transferred to the extraction tubes, and mixed with 15 ml of acetonitrile, 1 g of $MgSO_4$ and 0.25 g of NaCl. The mixture was shaken vigorously for 1 min and centrifuged (4500 rpm, 10 min). Afterwards, 12 ml of the supernatant was transferred to the 15ml clean tube, and mixed with 25 mg of PSA, 25 mg of C18 and 150 mg of $MgSO_4$. After centrifugation (4500 rpm, 10 min), 10 ml of supernatant was collected, dried under gentle nitrogen atmosphere, and redissolved in 1 ml of acetonitrile/milli-Q water (v/v, 7/3). The supernatant was injected into the LC-MSMS after centrifugation (4500 rpm, 10 min). Quechers showed a recovery of 42 to 111% with a standard deviation up to 15% (Table S2.2). Bioaccumulation was evaluated by the ratio between the amount of bioaccumulated OMPs and the initial amount of OMPs.

### 2.2.5 Analytical methods

The growth of *S. obliquus* in the treatments (A, B) and the controls (E, F) was quantified by measuring the chlorophyll a and the dry weight. The chlorophyll a was measured daily in duplicate using the PhytoPAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The dry weight was measured in duplicate at the end of the experiments (day 8) according to a standard method (Rice and American Public Health Association, 2012).

OMPs in the medium were analysed daily using the LC-MSMS (1290 infinity II with bubo QQQ, Agilent, Santa Clara, USA). Microalgal biomass samples were centrifuged (4500 rpm, 4 min). The supernatant was collected and diluted ten times with a mixture of BG-11 medium and acetonitrile. The column used was Zorbax plus C18 RRHT column (2.1*50 mm, 1.8 μm, P.N 827700-902, Agilent, Santa Clara, USA). The mobile phase included eluent A (milli-Q water with 0.1% formic acid) and eluent B (acetonitrile with 0.1% formic acid). The step gradient was set as below: 0-3 min constant at 10% B; 3 -20 min linearly increased to 60% B, 20-21min linearly increased to 100% B, 21-28 min constant at 100% B. The flow rate was kept at 0.2 ml/min in the first 2 min, then elevated to 3.0 ml/min. The column temperature was 25 °C. The injection volume of samples was 5 μL. DCF (296.0 -> 214.0), CLA (748.5 -> 158.0), BTZ (120.0 -> 65.3), CBZ (237.0 -> 194.0) and MET (268.0 -> 116.0) were detected in positive ionisation model, and MCPP (213.0 -> 141.0) was detected in negative ionisation mode.



### 2.2.6 Enzymatic activity assay

The activity of peroxidase (POX) at the end of the experiments (day 8) was determined by POX assay kits (Sigma-Aldrich, The Netherlands). One unit of POX activity was defined as the amount of enzyme that reduces 1 μmol $H_2O_2$ per minute at 37 °C. The POX activity was measured in treatment A with different OMPs, control E, and control F with BTZ and the mixture of OMPs. A microalgal sample (5 ml) was first centrifuged for 10 min at 4500 rpm at 4°C. The supernatant was used to determine extracellular enzymatic activity following the instructions of the manufacturers, and the pellet was used to determine the intracellular enzymatic activity. The pellet was suspended in 1 ml of Tris-HCl (pH = 7.4) solution after washing three times with milli-Q water. The suspension was then vortexed for 30s, sonicated for 5 min (Brenson sonifier 450P, ultrasonic time 10 s, rest time 10 s) to break down the microalgal cells, and centrifuged (10 min, 45000 rpm, 4 °C). Finally, the supernatant was used for the intracellular enzyme assay.

### 2.2.7 Statistical analysis

A Wilcoxon test was performed to identify the statically significance of dry weight and the POX activity between the treatments and controls.

## 2.3 Results and discussion

### 2.3.1 Microalgal growth

The chlorophyll a showed similar trends in the growth curves for all the OMPs, except for the one with CLA (Figure 2.2a). When BTZ, CBZ, MET, DCF and MCPP were present as individual compounds, similar dry weight at day 8 (1.6 ± 0.1 g/l, Figure 2.2b) and specific growth rate (1.0 ± 0.1 $d^{-1}$, Table S2.3) were achieved. This shows that BTZ, CBZ, MET, DCF and MCPP did not significantly affect microalgal growth.

In the presence of CLA, a lower dry weight and chlorophyll a was observed with *S. obliquus* at day 8 (Figure 2.2), indicating that the growth of *S. obliquus* was inhibited by CLA. In comparison, this species was less tolerant to CLA than *S. quadriculata*, *C. vulgaris* and *R. subcapitata* (Guo et al., 2020a; Kiki et al., 2020). So far, the inhibition processes of CLA on microalgal growth are unclear. It has however been described that CLA can inhibit the growth of bacteria by adversely affecting the synthesis of polypeptides and the translocation of aminoacyl transfer RNA (Guo et al., 2020b). During this process, CLA can inhibit the activity of enzymes such as P450, which is involved in the biodegradation of OMPs (Masubuchi and Horie, 2007; Akiyoshi et al., 2013). A similar process might occur in *S. obliquus*, since microalgae have similar OMPs degradation pathways as bacteria (Méndez García and García de Llasera 2021).

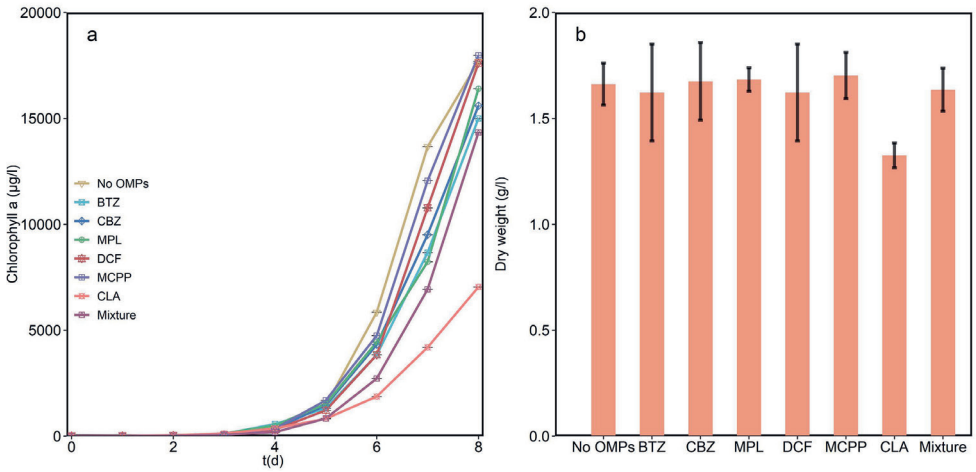



**Figure 2.2** Dry weight at day 8 (a) and chlorophyll a (b) in time in the presence of OMPs (treatment A) and the absence of OMPs (control E) with *S. obliquus*.

The dry weight at day 8 and specific growth rate in the presence of the mixture of OMPs were similar with control E (no OMPs) (Figure 2.2, Table S2.3). This shows that the mixture of OMPs, including CLA, did not inhibit microalgal growth. Furthermore, the mixture of OMPs had an antagonistic effect on the CLA-induced inhibition on microalgal growth. Sharma et al. (2021) showed that OMPs competed for the binding sites of cells responsible for growth inhibition. Possibly such a mechanism explains the effects of CLA on microalgal growth.

## 2.3.2 Removal processes

Three out of six compounds (DCF, CLA and BTZ) were 48 to 99% removed in the microalgae cultivation spiked with OMPs exposed to light (treatment A) (Figure 2.3). In contrast, CBZ, MET and MCPP showed negligible removal (Figure S2.2). Only a small amount (< 5%) of these three compounds was bioadsorbed or bioaccumulated by the microalgal biomass (Table S2.5).

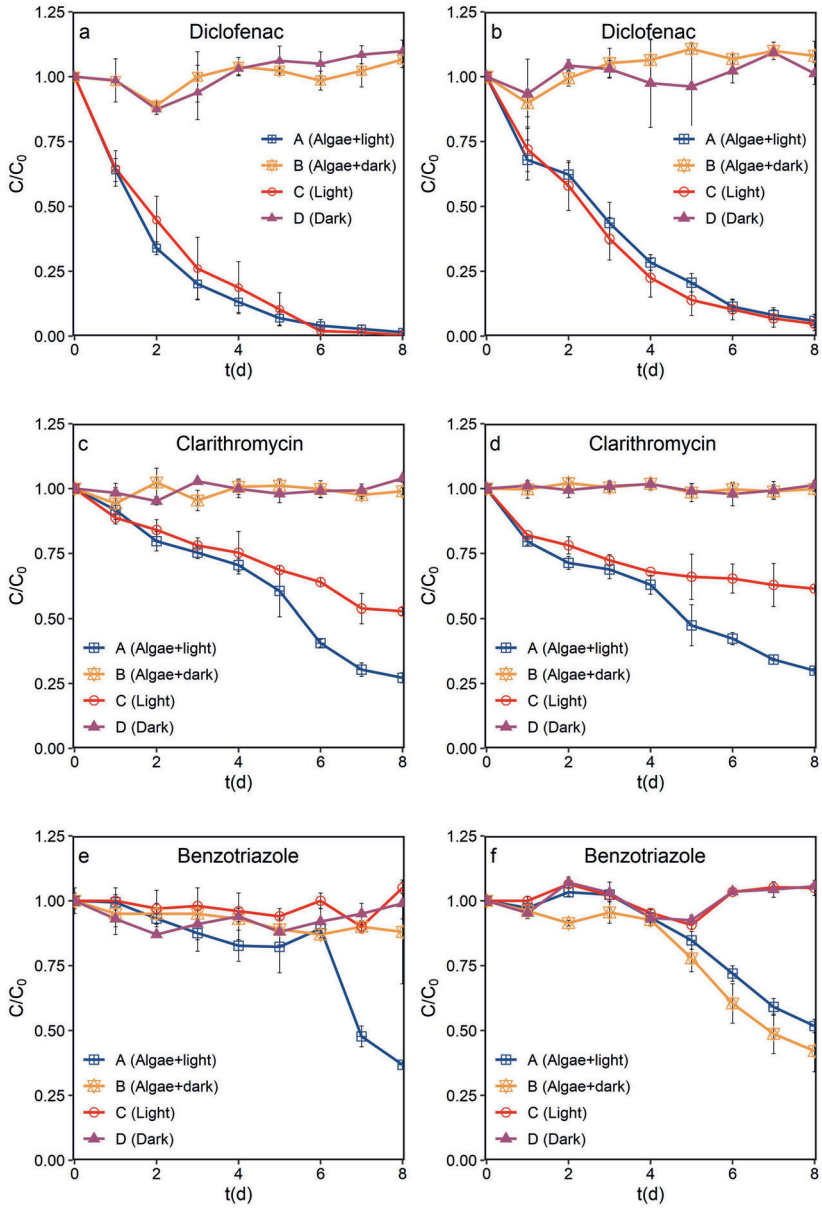



**Figure 2.3** Relative removal ($C/C_0$) of DCF (a), CLA (c) and BTZ (e) when present as individual compound; DCF (b), CLA (d) and BTZ (f) when present in the mixture.

DCF was only removed in the presence of light, with no significant difference in the presence or absence of microalgae (Figure 2.3a, b), indicating that photodegradation was the dominating removal process. An insignificant increase of DCF concentration was observed in control B (algae + dark) and D (Dark) due to minor errors in the LC measurements. In treatment A (algae + light), 99% of DCF was removed by photodegradation when present as individual compound, and 94% was photodegraded when present in the mixture of OMPs (Figure 2.4). It is known that DCF is a light sensitive compound in many aqueous media (He et al., 2016; Kanakaraju et al., 2016) due to the photosensitive nature of its chlorinated aromatic ring (Moore et al., 1990), and has shown different photodegradation efficiencies under varying environmental conditions, such as light intensity and growth medium composition (De Wilt et al., 2016; Nguyen et al., 2020). Previous work showed that 40 to 60% of DCF was removed by photodegradation in anaerobically digested black water (AnBW) in one month (De Wilt et al., 2016). Compared to our experiments, less continuous irradiation (80 µmol $m^{-2}$ $s^{-1}$ instead of 126 µmol $m^{-2}$ $s^{-1}$), indicating that irradiation optimization results in a high removal of individual light sensitive OMPs.



DCF biodegradation was not observed in our experiments (Figure 2.3a, b), which is in line with previous results (De Wilt et al. 2016). However, this contrasts other studies, reporting *Picocystis sp.* and *Graesiella sp.* to biodegrade 20 to 70% of DCF when present in high concentrations of 25 to 200 mg/l (Ben Ouada et al. 2019). Possibly, a concentration far above the mg/l range is needed to stimulate DCF biodegradation.

Bioadsorption and bioaccumulation of DCF were negligible (<1.2%). Likewise, another green microalgal species (*Chlollera sp.*) showed 5.5 to 7.5% of sorption (bioadsorption and bioaccumulation) of DCF when adding 147 µg/l of DCF in urine and AnBW (De Wilt et al., 2016).

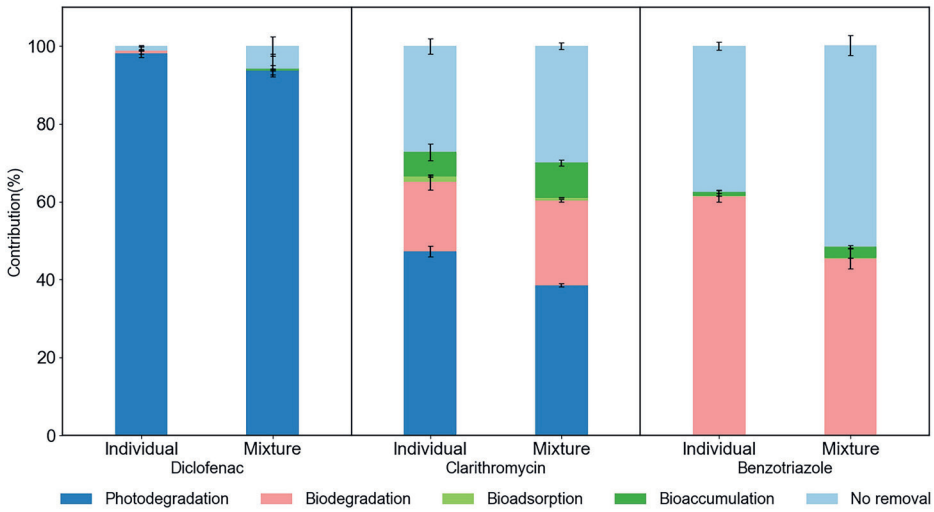



**Figure 2.4** Contribution of each removal process in treatment A at day 8.

CLA removal showed similar patterns in treatment A (algae + light) and control C (light) for the first four days (Figure 2.3c, d). This indicates that photodegradation dominated the removal of CLA during the first four days. Moreover, biodegradation, bioadsorption and bioaccumulation may contribute to an enhanced CLA removal in treatment A four days later (Figure 2.3c, d). Photodegradation of CLA accounted for 48% removal when present as individual compound, and 39% removal when present in the mixture of OMPs (Figure 2.4). This shows that CLA was less photodegradable than DCF, which is consistent with another study, focusing on the photodegradation at 10-cm depth in a constructed wetland basin (Mathon et al., 2019). In addition, indirect photodegradation of CLA can play a role in our experiments due to the presence of $Fe^{3+}$ in our medium. $Fe^{3+}$ is known to enhance indirect photodegradation via the formation of a $Fe^{3+}$- clarithromycin complex, which is a photochemically active compound (Kari and Giger, 1995; Vione et al., 2009; Mathon et al., 2019). When $Fe^{3+}$ and light were present, hydrogen peroxidase, produced by microalgae, could also contribute to CLA removal via the photo Fenton reaction (Karaolia et al., 2014; Vo et al., 2020).

Biodegradation was the second prominent removal process of CLA in treatment A. Biodegradation accounted for 27% removal of CLA as individual compound or in the mixture of OMPs (Figure 2.4). In batch systems with BG -11 medium, 75% of CLA was biodegraded by *H. pluvialis*, *S. capricornutum*, or *C. vulgaris* in 40 days (Kiki et al., 2020). Hydroxylation is an important pathway of CLA biodegradation in lettuce tissues (Tian et al., 2019). During the hydroxylation, cytochrome P450 enzymes add the hydroxyl group on the cladinose ring (Xiong et al., 2018; Tian et al., 2019). Possibly, similar pathway is involved in CLA biodegradation by microalgae.



Bioadsorption contributed to less than 1.5% to the removal of CLA, and bioaccumulation accounted for less than 10% removal of CLA (Figure 2.4). CLA showed the highest bioaccumulation in comparison with the other five OMPs. Bioaccumulation is affected by the hydrophobicity and the charge of OMPs (Sutherland and Ralph, 2019; Xiong et al., 2021). CLA belongs to the group of hydrophobic and positively charged compounds, which can bind strongly to the lipids of algal cells by hydrophobic interaction and to negatively charged cellular polymeric substances by electrostatic attraction (Sutherland and Ralph, 2019; Bui and Choi, 2010).

BTZ removal was only observed in the presence of algae (Figure 2.3e, f). In treatment A (algae + light), bioadsorption and bioaccumulation accounted for less than 3% removal (Figure 2.4). This indicates that biodegradation was the dominating removal process. BTZ was mainly removed by biodegradation when present as individual compound (63%) or in the mixture of OMPs (48%) (Figure 2.4). Biodegradation contributed to up to 50% of BTZ removal in a batch system with *Chlorella sp.* grown on Bold Basal Medium (Gatidou et al., 2019). In a high rate algal pond fed with urban wastewater, biodegradation contributed to 33 to 84% removal of BTZ (Matamoros et al., 2015). Additionally, in our experiments, the first-order kinetic constant of BTZ biodegradation when present as individual compound ($k_1 = 0.44\ d^{-1}$) was higher than in the mixture of OMPs ($k_1 = 0.14\ d^{-1}$) (Table 2.1). Asimakopoulos et al. (2013) found that BTZ was converted to 4- OH-BTZ by adding hydroxyl group on the triazole ring in BTZ biodegradation, and the conversion was mediated by P450 enzymes. Possibly, the lower BTZ biodegradation and first-order

kinetic constant in the mixture of OMPs in our experiments are due to the competition with CLA on the binding sites of P450 enzymes. However, the mixture of OMPs did not decrease the CLA biodegradation. The biomass in the mixed systems was 20% higher than in the individual CLA treatments (Figure 2.2b), most likely compensating this competitive inhibition effect.

In treatment B (algae + dark), BTZ removal was only achieved in the mixture of OMPs (Figure 2.3f). Apparently, a light-independent BTZ biodegradation occurred, which requires the presence of the other OMPs.

MET removal was not observed in our study, which is in line with previous findings (Bodin et al., 2016; García-Galán et al., 2020a). On the contrary, a mixed green algal population dominated by *Tetradesmus dimorphus* showed a complete degradation of MET in a pilot-scale open bioreactor (Gentili and Fick, 2017). Possibly, MET removal is species-dependent in microalgae-based systems. It can also be that bacteria played a role in those experiments, since these are known to degrade MET (He et al., 2018). In our experiments no bacteria were added.

MCPP was not removed in microalgae-based systems of this study and others (Matamoros and Rodríguez, 2016). In contrast, when an immobilised microalgae-based system was used, 70% biodegradation of MCPP can be achieved, due to the enhanced exchange of MCPP between the immobilized algae and bacteria (Ferrando and Matamoros, 2020).

CBZ was recalcitrant in our batch experiments, like many studies have also reported for microalgae-based systems (De Wilt et al., 2016; Matamoros et al., 2016; Larsen et al., 2019). In contrast, 30% of CBZ in BBM medium was removed in batch systems with *S. obliquus* for 10 days at 45 mmol/$m^2$.s at 27°C (Xiong et al., 2016). Possibly, a higher temperature and longer exposure time can induce CBZ biodegradation. The positive effect of the temperature was shown in a high rate algal pond, where CBZ biodegradation in the warm season was 30% higher than in the cold season (Matamoros et al., 2015).

## 2.3.3 Removal kinetics

The removal kinetics of DCF, CLA, and BTZ were investigated to elucidate the removal rate for various active processes (Table 2.1), using regression analyses on selected data presented in Figure 2.3.

**Table 2.1** Kinetic data of DCF, CLA or BTZ removal (n = 3).

| OMPs | Conditions | As individual compound | | In the mixture | |
|---|---|---|---|---|---|
| | | $k_1(d^{-1})$ | $R^2$ | $k_1(d^{-1})$ | $R^2$ |
| DCF | A (Algae + light) | 0.52 | 0.99 | 0.36 | 0.98 |
| | B (Algae + dark) | - | - | - | - |
| | C (Light) | 0.68 | 0.95 | 0.40 | 0.99 |
| | D (Dark) | - | - | - | - |
| CLA | A (Algae + light) | 0.18 | 0.93 | 0.15 | 0.97 |
| | B (Algae + dark) | - | - | - | - |
| | C (Light) | 0.08 | 0.97 | 0.04 | 0.94 |
| | D (Dark) | - | - | - | - |
| BTZ | A (Algae + light) | 0.44* | 0.95* | 0.14# | 0.98# |
| | B (Algae + dark) | - | - | 0.18# | 0.97# |
| | C (Light) | - | - | - | - |
| | D (Dark) | - | - | - | - |

# Data from day 3 to 8 were used.
* Data from day 6 to 8 were used.

DCF removal in control C (light) yielded a first-order kinetic constant ($k_1$) of 0.48 $d^{-1}$ in the presence as individual compound, which was higher than in the mixture of OMPs (0.40 $d^{-1}$) (Table 1). Similar with DCF in control C, CLA showed a faster removal when present as individual compound ($k_1$ = 0.08 $d^{-1}$) than in the mixture of OMPs ($k_1$ = 0.04 $d^{-1}$). Possibly, the photodegradation of DCF and CLA competed for the available photons. This effect has also been observed in other studies for solar

photodegradation of diclofenac and naproxen. However, this was at a higher incident light intensity (190 to 1900 µmol $m^{-2}$ $s^{-1}$) than our studies (126 µmol $m^{-2}$ $s^{-1}$) (Kanakaraju et al., 2016).

The first-order kinetic constant of BTZ removal in treatment A (algae + light) is the same order of magnitude as another lab batch system with *Chlorella sp*., with a $k_1$ of 0.11 $d^{-1}$ (Gatidou et al., 2019). In comparison with microalgae, activated sludge from a conventional WWTP showed a $k_1$ from 0.014 to 0.69 $d^{-1}$ for biodegradation of BTZ. BTZ removal in this study is within this range, indicating that the microalgae in our system can achieve a comparable biodegradation of BTZ. Additionally, the negligible bioaccumulation and bioaccumulation in microalgae-based system demonstrates that BTZ was removed from the system, instead of being transported to the microalgal biomass (Figure 2.4). A microalgae-based system therefore is a competitive alternative to conventional WWTP for BTZ removal.

### 2.3.4 Peroxidase activity

The activity of intracellular and extracellular POX was measured, as they can play an important role in the biodegradation of OMPs in microalgal cultures (Vo et al., 2020).

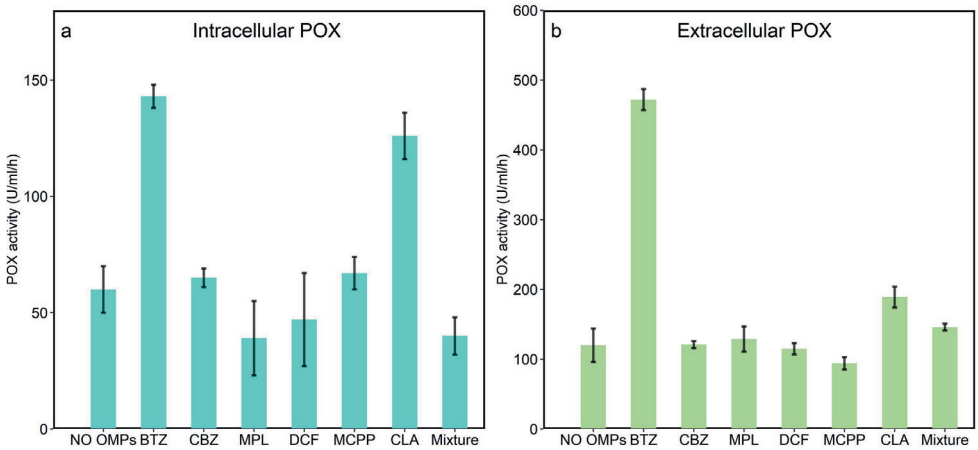



**Figure 2.5** Activity of intracellular (a) and extracellular (b) POX in the presence of OMPs (treatment A) and absence of OMPs (control E).

In treatment A (algae + light), the highest activity of intracellular and extracellular POX was found in the presence of BTZ as individual compound (Figure 2.5). Apparently, the presence of the mixture of OMPs reduced the production of intracellular and extracellular POX.

POX was reported to metabolise BTZ and its methyl derivatives (Wu et al., 1998). Vo et al. (2020) demonstrated that intracellular and extracellular POX were involved in the biodegradation of OMPs (tetracycline, sulfamethoxazole and bisphenol A) by *Chlorella sp.*. Another study identified that the genes encoding for POX were one of the key functional genes for enrofloxcin biodegradation by ryegrass (Zhao et al., 2021). In our study, the extracellular POX activity in treatment B (algae + dark) with BTZ in the mixture of OMPs was 75 U/ml/h, four-fold higher than when present as individual compound (18 U/ml/h). This is in line with high BTZ removal in treatment B with the mixture of OMPs (Figure 2.3). This shows that POX plays a role in BTZ biodegradation. The intracellular and extracellular POX were responsible for BTZ biodegradation in treatment A when present as individual compound.

In treatment A, the activity of intracellular and extracellular POX when CLA was present as individual compound was higher than in the mixture of OMPs (Figure 2.5). The increase of intracellular POX activity was also observed in *R. subcapitata* and *C. vuglaris* after exposure to higher than 20 µg/l of CLA (Guo et al., 2020a). However, CLA biodegradation was similar in treatment A with the presence of CLA as individual compound and in the mixture of OMPs (Figure 2.4). POX can not only use OMPs as substrate, but also use asorbate and glutathione, which are important antioxidants in algae cells (Nicodemus et al., 2020). Possibly, POX did not contribute to CLA biodegradation, but the oxidation of ascorbate and glutathione.

# 2.4 Conclusion

Photodegradation and biodegradation were the prevailing removal processes of DCF, CLA and BTZ in our experiments. For the other three OMPs (MET, MCPP and CBZ), no significant removal was achieved during the 8-day experiment by these two removal processes. Only a small fraction (< 5%) was removed due to bioadsorption and bioaccumulation. DCF was completely removed by photodegradation, and BTZ was mainly removed by biodegradation. For CLA, photodegradation dominated the removal during the first four days, and biodegradation started from day 4 onwards.

Inhibitory effects of the mixture of OMPs on photodegradation and biodegradation were identified in our system. The mixture of OMPs inhibited photodegradation, which is indicated by the lower first-order kinetic constants of photodegradation of DCF and CLA, when present in the mixture of OMPs. The adverse effect of the mixture of OMPs on BTZ biodegradation is manifested by the decreased biodegradation and decreased the first-order kinetic constant in the presence of the mixture of OMPs. No inhibitory effect of the mixture of OMPs was observed for CLA biodegradation.

POX participated in intracellular and extracellular biodegradation of BTZ when present as individual compound. POX did not participate in CLA biodegradation.

To conclude, the reactive OMPs (DCF, CLA and BTZ) are mainly photodegraded and biodegraded, while recalcitrant OMPs (MET, MCPP and CBZ) are bioadsorbed and bioaccumulated.



# Acknowledgements

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# SUPPLEMENTARY MATERIAL TO CHAPTER 2

**Table S2.1** The composition of BG-11 medium modified from Trebuch et al. (2020).

| **Macronutrients** | **Concentration (mg/l)** |
|---|---|
| $(NH_4)_2SO_4$ | 470 |
| $K_2HPO_4$ | 56 |
| $MgSO_4.7H_2O$ | 75 |
| $CaCl_2.\ 2H_2O$ | 36 |
| EDTA ferric sodium salt | 8.4 |
| $Na_2EDTA.\ 2H_2O$ | 1.8 |
| **Micronutrients** | **Concentration (mg/l)** |
| $H_3BO_3$ | 2.86 |
| $MnCl_2.\ 4H_2O$ | 8.1 |
| $ZnSO_4.\ 7H_2O$ | 0.44 |
| $CuSO_4.\ 5H_2O$ | 0.079 |
| $Na_2MoO_4.\ 2H_2O$ | 0.22 |
| $Co(NO_3)_2\ .6H_2O$ | 0.05 |
| **Buffer** | **Concentration (g/l)** |
| HEPES | 23.83 |

**Table S2.2** The internal standards and recoveries of OMPs in SPE and Quechers.

| OMPs | Internal standards | Recovery (%) | | | |
|---|---|---|---|---|---|
| | | Individual compound | | Mixture | |
| | | SPE | Quechers | SPE | Quechers |
| BTZ | BTZ-D4 | 100± 7 | 121 ± 5 | 73 ± 7 | 98 ± 15 |
| CLA | CLA-D3 | 125 ± 12 | 117 ± 11 | 81 ± 12 | 87 ± 15 |
| DCF | DCF-D4 | 54 ± 4 | 61 ± 7 | 54 ± 5 | 50 ± 5 |
| MET | MET-D7 | 75 ± 2 | 62 ± 6 | 53 ± 1 | 54 ± 7 |
| CBZ | CBZ-13C6 | 45± 3 | 74 ± 6 | 88 ± 3 | 68 ± 5 |
| MCPP | MCPP-D3 | 76 ± 12 | 57 ± 2 | 42 ± 0 | 111 ± 11 |

**Table S2.3** The specific growth rate and yield of *S. obliquus* in treatment A (with OMPs) and control E (no OMPs).

| OMPs | Specific growth rate($d^{-1}$) | Yield |
|---|---|---|
| No OMPs | 1.0±0.1 | 0.5 - 0.7 |
| BTZ | 1.0±0.1 | 0.5- 0.7 |
| CBZ | 1.0±0.1 | 0.5 - 0.7 |
| MET | 1.0±0.1 | 0.5 - 0.6 |
| DCF | 1.0±0.1 | 0.5 - 0.7 |
| MCPP | 1.0±0.1 | 0.5 - 0.7 |
| CLA | 0.9±0.0 | 0.4 – 0.5 |
| Mixture | 0.9±0.1 | 0.5 - 0.6 |

**Table S2.4** Statistical analysis results of dry weight at day 8.

| Mechanism | Comparison | p |
|---|---|---|
| Dry weight | BTZ vs No OMPs | 0.92 |
| | CBZ vs No OMPs | 0.75 |
| | MET vs No OMPs | 0.75 |
| | DCF vs No OMPs | 0.60 |
| | MCPP vs No OMPs | 0.60 |
| | CLA vs No OMPs | 0.027 |
| | Mixture vs No OMPs | 0.60 |

**Table S2.5** Bioadsorbed and bioaccumulated fractions (%) of OMPs at day 8.

| OMPs | As individual compound | | In the mixture | |
|---|---|---|---|---|
| | Bioadsorbed | Bioaccumulated | Bioadsorbed | Bioaccumulated |
| DCF | n.d. | n.d. | <0.1 | 0.4 ± 0.0 |
| CLA | 1.4 ± 0.3 | 5.2 ± 0.6 | 0.7 ± 0.2 | 9.2 ± 0.2 |
| BTZ | 0.1 ± 0.0 | 1.2 ± 0.4 | 0.2 ± 0.1 | 2.8 ± 0.4 |
| MET | 1.9 ± 0.3 | 0.1 ± 0.0 | 0.6 ± 0.1 | 4.9 ± 0.6 |
| CBZ | 4.8 ± 0.6 | 0.3 ± 0.1 | 1.6 ± 0.0 | 1.8 ± 0.2 |
| MCPP | 1.4 ± 0.2 | n.d. | 4.2 ± 0.0 | 1.0 ± 0.1 |

n.d. = not detected.

**Table S2.6** Statistical analysis results of intracellular and extracellular POX activity.

| Mechanism | Comparison | P |
|---|---|---|
| Intracellular POX | BTZ vs No OMPs | $2.2*10^{-4}$ |
| | CLA vs No OMPs | $5.9*10^{-3}$ |
| | DCF vs No OMPs | 0.32 |
| | mixture vs No OMPs | $6.1*10^{-2}$ |
| Extracellular POX | BTZ vs No OMPs | $1.6*10^{-6}$ |
| | CLA vs No OMPs | $8.2*10^{-2}$ |
| | DCF vs No OMPs | 0.17 |
| | mixture vs No OMPs | $1.1*10^{-2}$ |

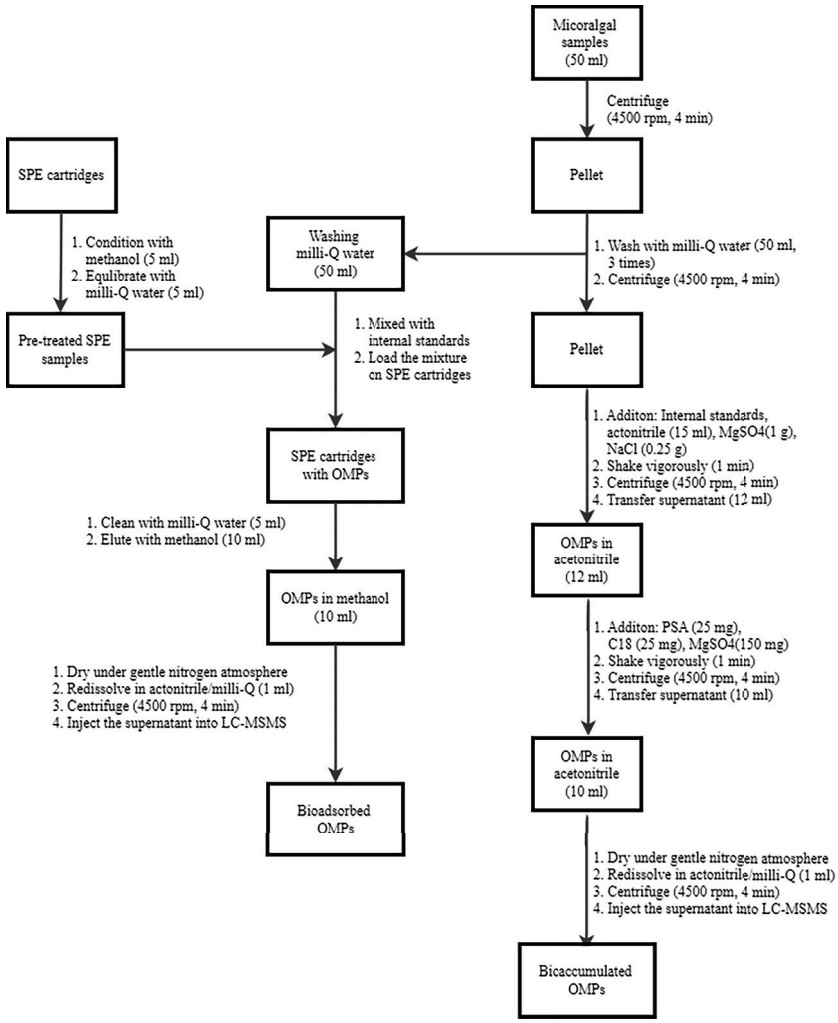



**Figure S2.1** Work-flow of extracting bioadsorbed and bioaccumulated OMPs.

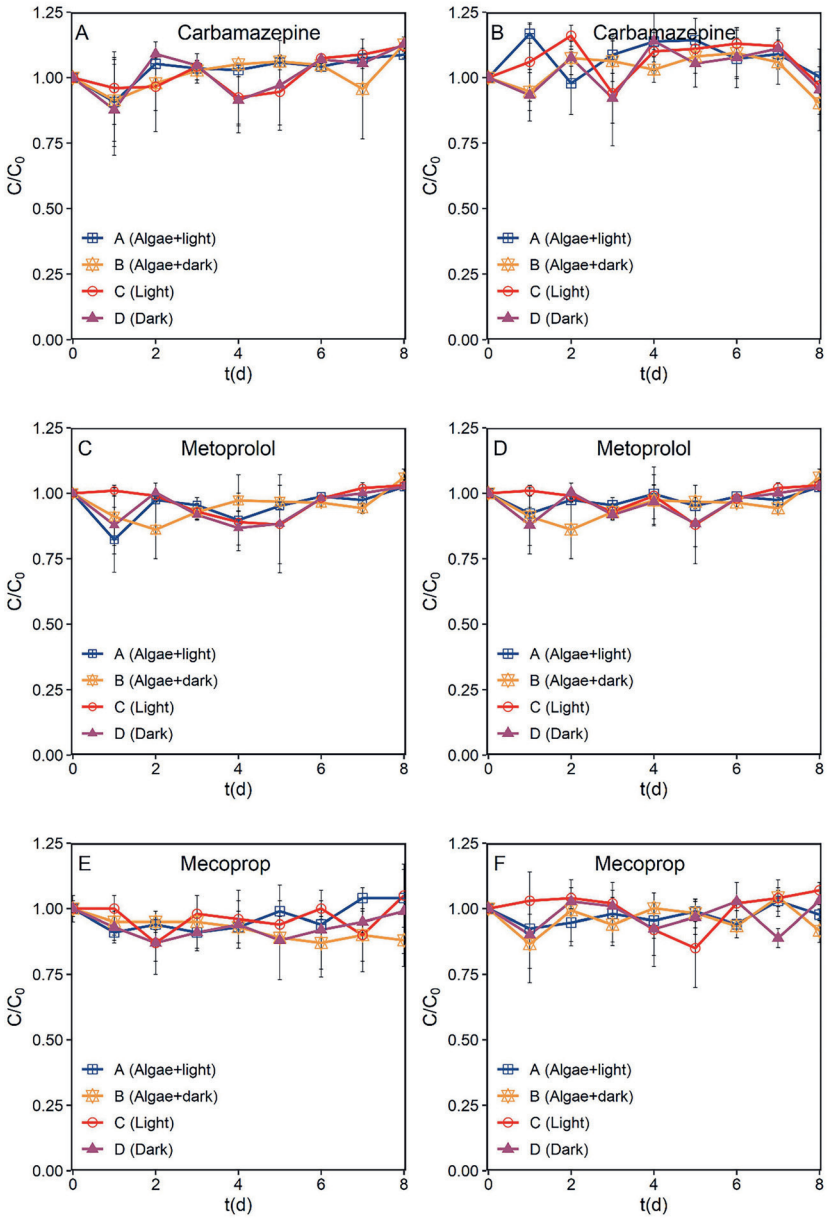
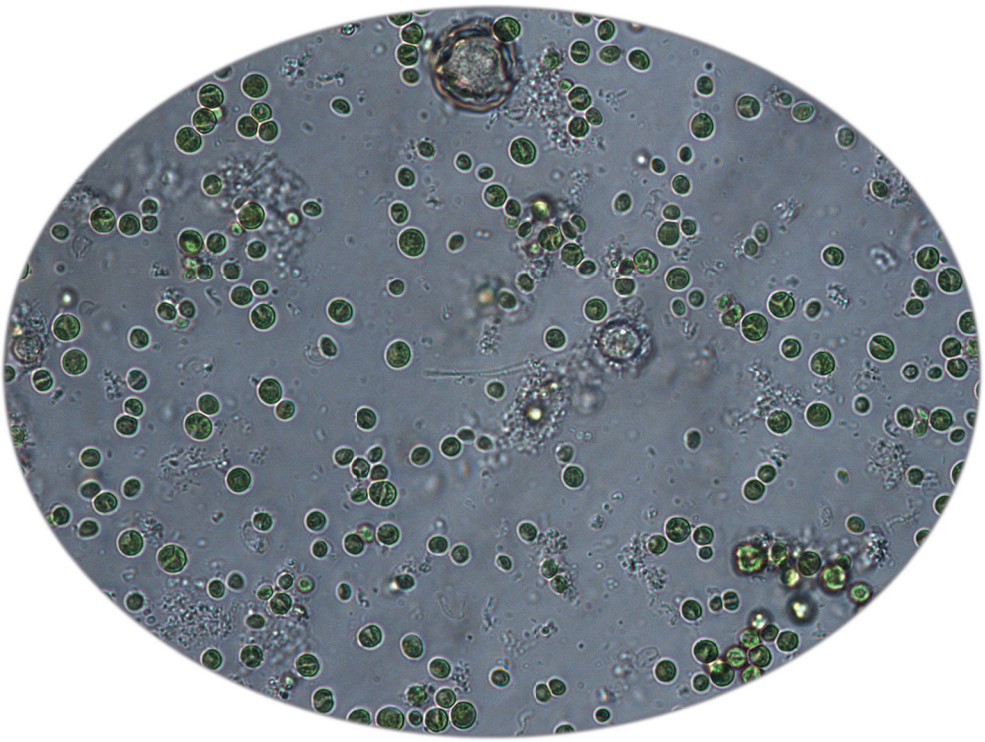



**Figure S2.2** Relative removal ($C/C_0$) of CBZ (A), MPL (C) and MCPP (E) when present as individual compound; CBZ (B), MPL (D) and MCPP (F) when present in the mixture.



# Chapter 3

# Impact of wastewater characteristics on the removal of organic micropollutants by *Chlorella sorokiniana*

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# Abstract

Microalgae-based technologies can be used for the removal of organic micropollutants (OMPs) from different types of wastewater. However, the effect of wastewater characteristics on the removal is still poorly understood. In this study, the removal of sixteen OMPs by *Chlorella sorokiniana*, cultivated in three types of wastewater (anaerobically digested black water (AnBW), municipal wastewater (MW), and secondary clarified effluent (SCE)), were assessed. During batch operation mode, eleven OMPs were removed from AnBW and MW. When switching from batch to continuous mode (HRT: 0.8 days), the removal of most OMPs from AnBW and MW decreased, suggesting that a longer retention time enhances the removal of some OMPs. Most OMPs were not removed from SCE since poor nutrient availability limited *C. sorokiniana* growth. Further correlation analyses between wastewater characteristics, biomass and OMPs removal indicated that the wastewater soluble COD and biomass concentration predominantly affected the removal of OMPs. Lastly, carbon uptake rate had a higher effect on the removal of OMPs than nitrogen and phosphate uptake rate.

These data will give an insight on the implementation of microalgae-based technologies for the removal of OMPs in wastewater with varying strengths and nutrient availability.

**Keywords:** emerging contaminants, microalgae technologies, wastewater strength, dry weight reduction, redundancy dimensional analysis

# 3.1 Introduction

Organic micropollutants (OMPs) are only partially removed by conventional wastewater treatment plants (WWTP), resulting in their accumulation into surface water (Joss et al., 2008; Rogowska et al., 2020). Since many OMPs, consisting of pharmaceuticals, personal care products and pesticides, are biologically active and persistent, they can negatively affect aquatic organisms, such as zooplankton, aquatic vertebrates and invertebrates (Zhang et al., 2020; Bertrand-Krajewski et al., 2022). It is therefore crucial to develop technologies for removing OMPs from wastewater, that are effective and sustainable (i.e. low requirement of energy and materials). Microalgae-based technologies fit these requirements (Sutherland and Ralph, 2019; Liu et al., 2021).



Microalgae-based technologies can efficiently remove a wide range of OMPs from different types of wastewater under various environmental conditions (Nguyen et al., 2020; Liu et al., 2021). In batch experiments where *Chlorella sorokiniana* was grown on diluted anaerobically digested black water (AnBW; 683 mg $COD_{soluble}$/l; 24.5 mg $PO_4^{3-}$-P/l; 540 mg $NH_4^+$-N/l), 60 to 100% of metoprolol, paracetamol, diclofenac, and ibuprofen were removed (De Wilt et al., 2016). A pilot high rate algal pond (HRAP), inoculated with *Chlorella vulgaris*, removed 51 to 90% of ibuprofen, methylparaben, and oxybenzone from municipal wastewater (MW; 247.3 mg TOC/l; 1.4 mg $PO_4^{3-}$-P/l; 20.1 mg $NH_4^+$-N/l; 0.4 mg $NO_3^-$-N/l) under semi-batch mode (Škufca et al., 2021). Batch reactors inoculated with *Chlorella sp.* and *Scenedesmus sp.*, removed 99 and 95% of caffeine and ibuprofen respectively from mixed wastewater (25% urban wastewater + 75% groundwater; 60.3 mg $COD_{soluble}$/l, 0.4 mg $PO_4^{3-}$-P/l; 11.8 mg $NH_4^+$-N/l; 28.5 mg $NO_3^-$-N/l) (Matamoros et al., 2016). Ferrando and Matamoros (2020) showed that an immobilised microalgae-based system removed 64 to 94% of sulfamethoxazole and 43 to 73% of mecoprop from modified ground water (5 mg $PO_4^{3-}$-P/l; 200 mg $NO_3^-$-N/l) under continuous conditions. This shows that besides microalgae species, OMPs concentrations and operation conditions of reactors, wastewater characteristics might play an important role in the removal of OMPs.

Most studies on microalgae technologies for wastewater treatment focused on the removal of OMPs in one type of wastewater (Villar-Navarro et al., 2018; Mezzanotte et al., 2022; Rambaldo et al., 2022). However, only very few studies have investigated the effect of wastewater characteristics on OMPs removal (De Wilt et al., 2016). In our previous work where *Chlorella sorokiniana* was cultivated in batch bottles, it was shown that the dissolved organic matter (DOM) in AnBW can act as a photosensitizer, therefore inducing more ibuprofen removal by photodegradation than in artificial urine (De Wilt et al., 2016). DOM can also influence the OMPs removal via enhancing or supressing microalgal biodegradation (Gatidou et al., 2019; Wang et al., 2020; Xiong et al., 2020). For example, Wang et al. (2020) found that 0.3 g/l of glucose increased the removal of carbamazepine from 30 to 50% via the enhancement of carbamazepine biodegradation in the batch experiments with *Spirulina platensis*. Gatidou et al. (2019) showed that 1 g/l of sodium acetate in artificial medium decreased benzotriazole removal from 20 to 80% via the suppression of benzotriazole biodegradation in the batch experiments with *Chlorella sorokiniana*. Compounds with similar structures in wastewater DOM might also affect the OMPs removal by similar mechanisms. On the other hand, many studies on the removal of OMPs from wastewater using microalgae and microalgae-bacteria consortium technologies have been conducted under batch mode (Matamoros et al., 2016; Ding et al., 2020; Escudero, 2020). Biological processes in WWTP are operated under continuous or semi-continuous modes (Grandclément et al., 2017). Operational mode (batch, semi-batch or continuous mode) can remarkably affect the efficiency of microalgae-based OMPs removal (Ummalyma et al., 2018). Previous study showed that continuous mode (HRT: 2 to 8 days) achieved 50% higher removal of chlorpyrifos and pentachlorobenzene than batch mode for 14 days, when a microalgal consortium dominated by *Chlorella sp.* and *Scenedesmus sp.* was applied (Matamoros and Rodríguez, 2016). The authors proposed that the continuous mode of operation (HRT: 2 to 8 days) increased the contact time between OMPs and biomass, thus increasing the removal of OMPs. Therefore, understanding the removal of OMPs under continuous mode of operation is crucial for scaling up microalgae-based technologies for wastewater treatment and completing the picture of the potential of such technologies for OMPs removal.

In this study, the growth of *Chlorella sorokiniana* in three types of wastewater was followed and the effect of sixteen OMPs on its growth was assessed under batch and continuous mode of operation. Further, the removal efficiencies of these sixteen OMPs were investigated. Wastewater characteristics, biomass growth, and the overall removal of OMPs were correlated to elucidate which parameters predominantly affect the removal of OMPs.

# 3.2 Methods and materials

## 3.2.1 Cultivation medium



AnBW, MW and secondary clarified effluent (SCE) were selected as the cultivation media for the experiments since they have distinct characteristics in terms of soluble COD, nitrogen, phosphorus, and C/N/P molar ratio (Table 3.1). Dissolve inorganic nitrogen (DIN) in all wastewater only consist of ammonia and nitrate due to the absence of nitrite.

**Table 3.1** Average characteristics (± standard deviation) of all wastewater (n = 3).

| | **AnBW** | **MW** | **SCE** |
|---|---|---|---|
| pH | 10.1 ± 0.5 | 7.6 ± 0.2 | 8.5 ± 0.4 |
| $COD_{soluble}$ (mg/l) | 1570 ± 31 | 681 ± 5 | 31 ± 3 |
| TSS (mg/l) | 206 ± 21 | 168 ± 4 | < DL |
| VSS (mg/l) | 191 ± 19 | 158 ± 3 | < DL |
| Alkalinity (mg $CaCO_3$/l) | 875 ± 13 | 185 ± 2 | 94 ± 0 |
| TN (mg/l) | 1912 ± 18 | 83 ± 2 | 4 ± 0 |
| $NH_4^+$-N (mg/l) | 1291 ± 31 | 63 ± 3.1 | 0.2 ± 0.0 |
| $NO_3^-$-N (mg/l) | <DL | < DL | 2 ± 0 |
| DIN (mg/l) | 1291 ± 31 | 63 ± 3 | 2 ± 0 |
| TP (mg/l) | 152 ± 4 | 9 ± 0.2 | 0.2 ± 0.0 |
| $PO_4^{3-}$-P (mg/l) | 100 ± 4 | 8 ± 0.2 | 0.2 ± 0.0 |
| N/P molar ratio | 29/1 | 17/1 | 23/1 |

DL = Detection limit.

AnBW was collected from a UASB reactor treating vacuum-collected black water of a two-person household in Wageningen, The Netherlands. MW and secondary clarification effluent (SCE) were collected from the WWTP of Bennekom, The Netherlands. In this WWTP, municipal wastewater is treated by conventional activated sludge technology, followed by a settling tank and a sand filtration. Municipal wastewater refers to the influent of the WWTP, while secondary clarified effluent refers to the effluent of the settling tank.

After collection, all three types of wastewater were autoclaved at 121℃ for 90 minutes to remove potential human pathogen contamination. AnBW was further centrifuged at 4500 rpm for 5 min to remove suspended solids and prevent clogging of the tubings feeding the photobioreactors. All wastewater were stored at 4 ℃ under anaerobic conditions until use.

### 3.2.2 Microalgae species

*Chlorella sorokiniana* originated from the culture collection at the Netherlands Institute of Ecology (NIOO-KNAW), The Netherlands. It was maintained in M8a medium (Kliphuis et al., 2010) at 35 °C under continuous average irradiation of 80 μmol $m^{-2}$ $s^{-1}$.

### 3.2.3 Target OMPs

Sixteen OMPs were selected based on the diversity of therapeutical class, the measurability, the persistency in wastewater and aquatic ecosystems, and the guideline list of OMPs released by the Dutch Foundation for Applied Water Research (STOWA) (Giannakis et al., 2015; STOWA, 2020). The OMPs are caffeine (CAF), trimethoprim (TRI), propranolol (PRO), carbamazepine (CBZ), sulfamethoxazole (SUL), benzotriazole (BTZ), 4/5-methylbenzotriazole (MeBT), clarithromycin (CLA), irbesartan (IRB), metoprolol (MET), diclofenac (DCF), ibuprofen (IBU), furosemide (FUR), hydrochlorothiazide (HYD), mecoprop (MCPP), and 2-methyl-4-chlorophenoxyacetic acid (MCPA). The OMPs were spiked in wastewater according to Wu et al. (2022). We spiked each compound in the cultivation media to a final concentration of 6 μg/l. Caffeine was already present

in the non-spiked AnBW and MW, thus reaching a final concentration of respectively 183 ± 3 and 25 ± 0 µg/l.

### 3.2.4 Experimental set-up

Four 380 ml flat panel photobioreactors (PBRs) were inoculated with 133 ± 4 µg chlorophyll a/l of *C. sorokiniana*. Two replicate PBRs were fed with OMPs spiked wastewater (treatment reactors), while the other two replicate PBRs were fed with non-spiked wastewater (control reactors). Each PBR had a light path of 14 mm, and an illuminated area of 0.027 $m^2$. Optimal temperature (35 °C) and pH (6.8 ± 0.1) for the growth of *C. sorokiniana* were automatically controlled (Fernandes et al., 2015). The content of each PBR was homogeneously mixed by bubbling air enriched with 10% $CO_2$ at a flow rate of 400 ml/min. During the AnBW and MW experiments, the light regime followed a sinus curve with a maximum average light intensity (400 to 800 nm) of 150 µmol $m^{-2}$ $s^{-1}$ and a 16:8 (light: dark) cycle. In the SCE experiment, the maximum average light intensity was 100 µmol $m^{-2}$ $s^{-1}$ to prevent light inhibition on microalgal growth.

The PBRs were initially operated in batch mode until the end of the exponential growth phase of microalgae. Then a continuous mode of operation (HRT: 0.8 days) was applied until the end of the experiment, when a steady state was reached. Steady state is defined as the period when the dry weight and chlorophyll a are stable for a minimum of five consecutive days, with a maximum standard deviation up to 5%.

### 3.2.5 Analytical methods

Algal biomass was daily quantified by dry weight and chlorophyll a during the continuous mode of operation. Dry weight was measured by a standard method of Rice and American Public Health Association (2012), and chlorophyll a was measured by a PhytoPAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Both measurements were performed in duplicate.

The elemental composition of dried biomass was determined in duplicate during steady state. For the analysis of biomass C and N content, a dried biomass sample was placed into a small tin cup and measured in an organic elemental analyzer (Flash

2000, Interscience Breda). For the analysis of P, the dried biomass was combusted at 550 ℃ for 30 min and digested with 10 ml persulfate (2.5%) at 121 ℃ for 30 min. The digested supernatant was used for P measurement by a PhosVer® 3 Phosphate Reagent Powder Pillow (Hach Lange, The Netherlands). For dissolved inorganic nutrients ($NH_4^+$-N, $NO_3^-$-N, $NO_2^-$-N, and $PO_4^{3-}$-P), 2 ml of AnBW or MW samples, or 10 ml of SCE samples, were filtered with a 0.2 μm cellulose acetate filter (VWR, The Netherlands), diluted with demi water to a final volume of 10 ml, and measured using a Seal QuAAtro39 AutoAnalyzer (SEAL, Analytical Ltd., Southampton, UK). Prior to OMPs measurement, 5 ml of microalgal biomass samples were daily collected from the PBRs. After centrifugation (4500 rpm, 10 min), 3 ml of supernatant was used for solid phase extraction (Wu et al., 2022). Solid phase extraction recoveries of sixteen OMPs were 40 to 103% with a standard deviation up to 10% (Table S3.1).

OMPs, except for IBU and FUR, were measured by a liquid chromatograph coupled to a triple quadruple mass spectrometer (LC-MSMS) as described in Wu et al. (2022). CAF (transition; 195.0 -> 138.0), TRI (291.0->230.0), PRO (260.0 -> 116.0), CBZ (237.0 -> 194.0), SUL (254.0 -> 92.0), BTZ (120.0 -> 65.3), MeBT (134.0 -> 77.2), CLA (748.5 -> 158.0), IRB (429.2 -> 207.1), MET (268.0 -> 116.0), DCF (296.0 -> 214.0), and HYD (296.0 -> 268.8) were measured in positive ionisation mode, while MCPP (213.0 -> 141.0) and MCPA (199.0 -> 141.0) were measured in negative ionisation mode.

IBU and FUR were measured by an ultra-high performance liquid equipped with a tandem mass spectrometer as described in Van Gijn et al. (2021). IBU (205.0 ->161.2) and FUR (328.9 -> 285.0) were measured in negative ionisation mode.

The measurement error was 10% for MCPA and 5% for other OMPs in our study (data not shown). Thus, removal lower than 10% was regarded as negligible for MCPA, and lower than 5% for all other OMPs.

### 3.2.6 Statistical analyses

The statistical analyses were conducted to elaborate on the effect of wastewater characteristics ($COD_{soluble}$, DIN, and $PO_4^{3-}$-P), kinetic parameters (chlorophyll a, dry weight and growth rate), and nutrient (C/N/P) uptake rate of the biomass on the removal of OMPs during steady state. Since the removal of MET, CBZ, MCPP, MCPA, and DCF was negligible in all wastewater, these five OMPs were not included in the statistical analyses.

The nutrient uptake rate of the biomass was calculated based on the C/N/P ratios of biomass: the C/N/P ratios were 179/22/1 for AnBW, 265/26/1 for MW, and 675/26/1 for SCE, respectively. The Principal Component Analysis (PCA) showed the dimension reduction of the OMPs data. The first two principal components PC1 and PC2 were used to represent the OMPs removal data. To determine the limiting conditions on OMPs removal, the Redundancy Dimensional Analysis (RDA) was used with PC1 and PC2 of OMPs removal. Similarly, RDA was performed with the nutrient uptake rate of the biomass and PC1 and PC2 of OMPs removal. All the statistical tests were conducted using OriginPro, Version 2022b, OriginLab Corporation, Northampton, MA, USA.



## 3.3 Results and discussion

### 3.3.1 Microalgal growth

The experiments with *C. sorokiniana* were started in batch mode to achieve exponential growth and therefore high biomass. When growth rate decreased mostly due to nutrient depletion, continuous mode was applied therefore continuously supplying nutrients at an HRT of 0.8 days. After a few days steady state was achieved, indicating that the growth rate of *C. sorokiniana* was constant.

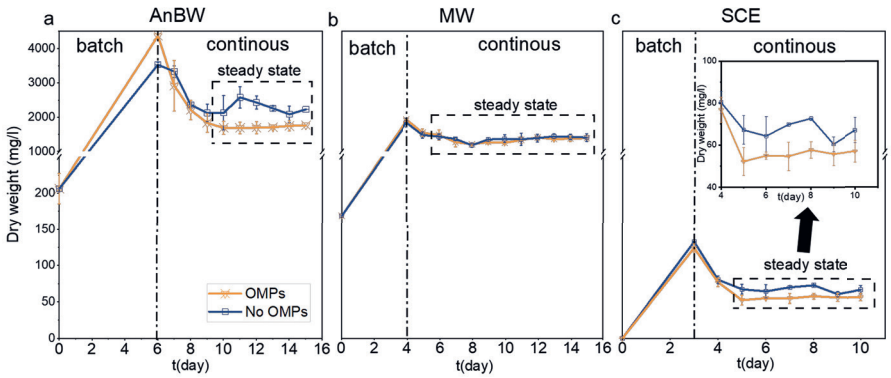


**Figure 3.1** Dry weight of *Chlorella sorokiniana* in time in AnBW (a), MW (b) and SCE (c).

The dry weight of *C. sorokiniana* in AnBW in the treatment (with OMPs) reached 4367 ± 42 mg/l at the end of the batch mode (day 6), and 1714 ± 32 mg/l in continuous mode during steady state (day 10 to 15) (Figure1a). In comparison with the control (no OMPs), the dry weight was 15% higher at the end of the batch mode, but 14% lower during steady state. A similar trend was observed for chlorophyll a, even though the standard deviation during steady state was much larger (10% for control, 11% for treatment, Figure S3.1a).

In MW, the dry weight was 1845 ± 131 mg/l at the end of the batch mode in the treatment and 1371 ± 75 mg/l during steady state of continuous mode (day 6 to 15). The dry weight and chlorophyll a were similar in both treatment and control (Figure 3.1b, S3.1b).

In SCE, a much lower dry weight and chlorophyll a was obtained than with AnBW and MW (Figure 3.1c, S3.1c) due to the low nitrogen and phosphorus concentrations in the medium (Table 3.1). During steady state (day 5 to 10), the dry weight in the treatment (573±14 mg/l) was 16% lower than control (4% standard deviation), while the chlorophyll a in the treatment was only 11% lower and with low deviation (1%).

OMPs positively affected the microalgal growth in batch mode in AnBW, but not in MW and SCE. During steady state, OMPs appeared to slightly inhibit the microalgal

growth in AnBW and SCE. This is difficult to validate due to the large standard deviation between control and treatment for both dry weight and chlorophyll a. This was not the case for MW, where clearly no inhibition was found. In batch mode, *C. sorokiniana* was exposed to the lower concentration of OMPs due to the higher removal efficiency in comparison with steady state in continuous mode (Figure 3.2). Mao et al. (2021) found that azithromycin stimulated the growth of *Chlorella pyrenoidosa* at low concentration (0.5, 1 µg/l), while inhibited the growth at high concentration (5 to 100 µg/l). Possibly, this stimulation at low concentration of OMPs also occurred in batch mode. During steady state, the higher concentration of OMPs inhibited the growth possibly by intervening the synthesis of protein in chloroplasts, as it has been demonstrated previously (Xiong, 2016; Miazek and Brozek-Pluska, 2019; Le et al., 2022). In MW, DOM may have reduced the bioavailability of OMPs by complexation of DOM and OMPs, and further mitigate the potential toxic effect of sixteen OMPs. Tong et al. (2020) showed that commercial DOM reduced the inhibition of tetracycline to *Coelastrella sp.* by the binding of tetracycline to the DOM. In SCE, the poor removal of OMPs in batch mode (Figure 3.2) resulted in a higher exposure concentration of OMPs than in AnBW, thereby allowing for a higher inhibitory effect on the removal of OMPs. These results indicate that the effect of OMPs on microalgal growth was influenced by both OMPs removal and DOM in wastewater.



### 3.3.2 OMPs removal

Generally, eleven out of sixteen OMPs were removed from AnBW and MW, except for MET, CBZ, MCPP, MCPA, and DCF (Figure 3.2). On the contrary, only CAF, IBU, PRO, CLA, and IRB (five out of sixteen) were removed from SCE.

| | AnBW | | MW | | SCE | |
|---|---|---|---|---|---|---|
| OMPs | Batch | Continous | Batch | Continous | Batch | Continous |
| CAF | 99 | 92 | 93 | 93 | 7 | 29 |
| BTZ | 88 | 57 | 89 | 69 | -3 | 0 |
| SUL | 91 | 79 | 76 | 83 | -3 | 3 |
| IBU | 92 | 56 | 75 | 71 | 11 | 32 |
| FUR | 90 | 38 | 69 | 74 | -3 | 4 |
| PRO | 82 | 51 | 90 | 64 | 5 | 83 |
| HYD | 69 | 18 | 29 | 3 | 0 | 0 |
| MeBT | 51 | 39 | 47 | 42 | 0 | 2 |
| CLA | 50 | 75 | 88 | 87 | 79 | 58 |
| TRI | 27 | 4 | 62 | 38 | 0 | 4 |
| IRB | 29 | 18 | 11 | 2 | 28 | 17 |
| MET | 9 | 2 | 4 | 2 | -1 | 2 |
| CBZ | -3 | 2 | 4 | -2 | -2 | 1 |
| MCPP | 0 | 3 | -3 | 0 | -2 | 2 |
| MCPA | 3 | -2 | 7 | 2 | -1 | 2 |
| DCF | 5 | 2 | 5 | 0 | 1 | 2 |

| | | | | |
|---|---|---|---|---|
| < 20 | 20 - 40 | 40 - 60 | 60 - 80 | 80 - 100 |

**Figure 3.2** Heatmap of OMPs removal (%) in AnBW, MW and SCE. The removal in batch mode refers to the removal at the end of batch mode (day 6, 4 and 3 for AnBW, MW and SCE, respectively). The removal in continuous mode refers to the average removal during steady state. The standard deviations of removal in batch and continuous mode are shown in Table S3.2.

The compounds (CAF, BTZ, SUL, IBU, FUR, and PRO) showed the highest removal (82 to 99%) in batch mode with AnBW, while a decrease was observed for all these compounds after switching to continuous mode, except for CAF for which removal remained almost 100%. In MW, the removal of these OMPs ranged from 69 to 93% in batch mode. In comparison with AnBW, BTZ and PRO removal decreased less, and the removal of other OMPs remained constant or showed limited increase (<7%) upon changing from batch to continuous mode. The observed decrease of the removal of OMPs from batch to continuous mode was paralleled with

a decrease of biomass, as lower biomass means less enzymes and less adsorption surface available for the removal of OMPs. A lower decrease in the removal of CAF, BTZ, SUL, IBU, FUR, and PRO was observed in MW from batch to continuous mode due to a lower decrease (25% in dry weight) of biomass than in AnBW (61% in dry weight). In SCE, the removal of most OMPs was negligible in batch mode, and upon switching to continuous mode, increased for three compounds (CAF, IBU, and PRO) to more than 20%. Most likely, the low biomass in SCE resulted in a negligible removal of most OMPs (Figure 3.1c), while for CAF, IBU and PRO, the removal capacities of *Chlorella sorokiniana* were enhanced by acclimatizing to these OMPs, even at low biomass concentrations (Hena et al., 2021). Additionally, aromatic compounds containing nitrogen, such as CAF and PRO, may serve as extra nitrogen sources for maintaining the growth of *Chlorella sorokiniana*, therefore leading to a remarkable removal of DIN in SCE. Luther (1990) found that *Scenedesmus obliquus* grew by using nitro- and ammonia substituted aromatic compounds (amino naphthalene, 4-amino naphthalene-1-sulfonic acid, 4-aminobenzoate, 4-nitroanilene, and 2-nitrobenzoate) as nitrogen sources in the absence of inorganic nitrogen sources. The removal efficiencies of these six OMPs in our experiments with AnBW and MW are within previously reported removal efficiencies in other microalgae-based systems, such as flasks and pilot-scale HRAP (Matamoros et al., 2015; Hom-Diaz et al., 2017; Villar-Navarro et al., 2018; Gatidou et al., 2019; Gojkovic et al., 2019; Ding et al., 2020; García-Galán et al., 2020a).

In comparison, HYD, MeBT, CLA, TRI, and IRB showed less removal in batch mode with AnBW. Furthermore, the removal of these OMPs in AnBW decreased after switching from batch to continuous mode, except for CLA, for which the removal increased. In MW, all these compounds showed a decrease of removal when switching from batch to continuous mode. HYD and IRB showed less removal than AnBW, and MeBT was removed similarly in both wastewater. Specially, CLA in batch mode with MW (88%) and SCE (79%) showed higher removal than AnBW. TRI removal in batch mode with MW was 35% higher than AnBW. Most likely, nutrient limitation (Figure S3.2) during the experiment with MW stimulated the production of peroxidase and P450 enzymes (Teng et al., 2019; Gauthier et al., 2020). These enzymes are responsible for TRI removal (Damsten et al., 2008; Almaqdi et al., 2019), and P450 enzymes can remove CLA by adding hydroxyl

group on its cladinose ring (Tian et al., 2019). A higher removal of CLA and TRI was therefore achieved in batch mode with MW.

MET, CBZ, MCPP, MCPA, and DCF, were poorly removed in all three types of wastewater. The poor removal efficiencies of MET, CBZ and MCPP were in line with previous studies (De Wilt et al., 2016; García-Galán et al., 2020a; Wu et al., 2022). MCPA has a similar recalcitrant structure as MCPP, which includes an aromatic ring with a carboxylic side chain (Parus et al., 2021). This might explain the poor removal of MCPA. Poor removal of MCPA in sewage was also observed in batch experiments with four different green algal species (*Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris*) under fluorescent light (Zhou et al., 2014). In contrast, 89% of MCPA removal in agricultural run-off was achieved in a full-scale semi-closed PBR inoculated with a mixed community of bacteria, microalgae, protozoa and small metazoan, under natural light conditions (García-Galán et al., 2020b). This removal was attributed to photodegradation and biodegradation. The photodegradation of MCPA required the light with wavelength of lower than 290 nm (Muszyński et al., 2020). MCPA therefore was not removed by photodegradation under visible light (400 to 800 nm) in our study. DCF removal on the other hand has been shown to be completely removed by photodegradation under white fluorescence light (De Wilt et al., 2016; Wu et al., 2022). Under natural light conditions, 20 to 60% of DCF removal was observed in multiple pilot-scale HRAP (Matamoros et al., 2015; García-Galán et al., 2020a; Vassalle et al., 2020). The contradicting results between this study and others are because visible light (400 to 800 nm) in this study is unable to induce DCF photodegradation (Rashid et al., 2020; John et al., 2021). Therefore, applying a light source with the same spectrum as sunlight can be a solution for optimising the removal of MCPA and DCF in this study.

### 3.3.3 Effect of wastewater characteristics and biomass composition on OMPs removal

RDA was applied to show that OMPs removal was influenced by wastewater type (MW, SCE and AnBW), as shown by wastewater characteristics, kinetic parameters of the reactors and biomass characteristics (Figure 3.3).

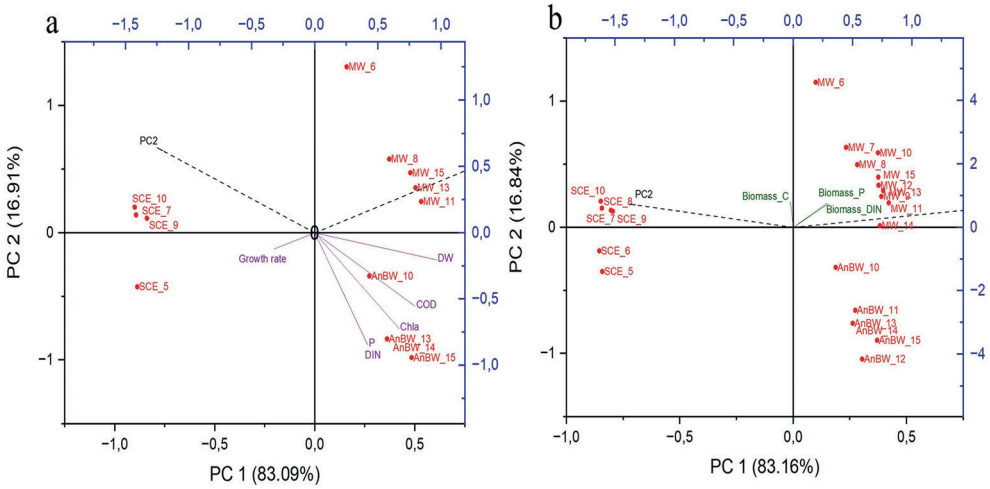


**Figure 3.3** RDA analysis of PC1 and PC2 of OMPs removal with wastewater characteristics and kinetic parameters (a), nutrient uptake rate of the biomass (b). Sampling points are indicated as red dots in the graphs. The wastewater characteristics and kinetic parameters are shown in rays. The angles between different rays represent their correlations, and a sharper angle shows a stronger correlation.

The coefficients of RDA showed that dry weight (RDA1: 0.96) and soluble COD (RDA1: 0.79) had the highest positive impact on the total removal of OMPs (Figure 3.3a), whereas the concentrations of DIN (RDA1: 0.41 and RDA2: -0.84) and $PO_4^{3-}$-P (RDA1: 0.41 and RDA2: -0.84) were not as effective. The similar tendencies in dry weight and the removal of most OMPs in AnBW and MW from batch to continuous mode manifested the positive impact of biomass concentration on the removal of OMPs. Therefore, increasing biomass levels in microalgae-based photobioreactors, either by increasing the HRT or by decoupling HRT from SRT, appears to be an important way to further optimize the removal of OMPs. Furthermore, soluble COD in wastewater can affect OMPs removal by complexation of DOM and OMPs in microalgae-based systems (Ding et al., 2018; Tong et al., 2020). DOM, such as humic acid, reduced the removal of triclosan by *Cymbella sp.* because the complexation of humic acid and triclosan reduced the availability of triclosan to microalgal cells for subsequent biodegradation (Ding et al., 2018). This

negative effect was also observed in the removal of tetracycline by *Coelastrella sp*. (Tong et al., 2020). These negative results contradicted with the outcome of our study. Possibly, other mechanisms induced the positive effect of soluble COD on OMPs removal. Humic substances in DOM can function as surfactant and emulsifier (Klavins and Purmalis, 2010), which can increase the accessibilities of OMPs to the biomass and the subsequent intracellular biodegradation (Sutherland and Ralph, 2019). Humic acid contains quinone moieties, and can act as electron shuttle to enhance the electron transfer between electron donors and electron acceptors (Martinez et al., 2013; Lipczynska-Kochany, 2018). He et al. (2018) found that this mechanism was responsible for enhancing the removal of metoprolol, naproxen, and diclofenac (electron donors) by DOM from constructed wetland in aerobic enrichment cultures. Due to the similarities of the removal pathways of OMPs by microalgae and bacteria (Méndez García and García de Llasera, 2021), this process can play a role in our microalgae-based systems.

The biomass uptake rate of C/N/P played a significant role in total removal of OMPs. The mole of C in the biomass (RDA2: 0.82) showed a higher impact than the mole of N (RDA2: 0.76) and $PO_4^{3-}$-P (RDA2: 0.74) on OMPs removal (Figure 3.3b). This is also shown by the variable importance plot (VIP) of each parameter on the total removal of OMPs (Figure S3.3b). The biomass in MW showed higher mole C uptake rate than AnBW (Table S3.3). This was probably because of the accumulation of carbohydrate and lipid in *Chlorella sp.*, induced by limited nutrient availability in MW (Gumbi et al., 2022). Conjunction with carbohydrate, such as glucose, is an important step of removal of some OMPs, such as IBU and BTZ (LeFevre et al., 2015; Marsik et al., 2017). Possibly, the accumulated carbohydrate accelerated this procedure and resulted in a higher removal. Carbohydrates, such as glucose, can also act as co-substrate for the removal of OMPs (e.g. tetracycline and bisphenol A) and lead to a higher removal in the batch experiments with *Chlorella sorokiniana* (Vo et al., 2020). However, the addition of 0.5 g/l glucose completely inhibited the removal of ciprofloxacin in flask experiments with *Chlamydomonas Mexicana* since the easily available carbon source (glucose) inhibited the synthesis of enzymes available for ciprofloxacin removal (Xiong et al., 2017b). This inhibitory effect of carbohydrate might also occur in the removal of some OMPs in our study. Overall,

a combination of these mechanisms can result in the significant impact of carbon uptake rate of the biomass to the overall removal of OMPs.

## 3.4 Conclusion

In batch mode, eleven out of sixteen OMPs were highly removed from AnBW and MW, whereas most OMPs showed poor removal (<11%) in SCE, except CLA and IRB. The removal of most OMPs decreased in AnBW and MW when the operation was switched from batch to continuous mode. However, removal percentages remained above 60% for most of these 11 OMPs. The reduced biomass concentrations during continuous mode seem to be the most important factor in this decrease in OMPs removal as less enzymes and adsorption surface are available for the removal of OMPs. An increase in the removal of CAF, IBU and PRO in SCE was observed when switching from batch to continuous mode. It appears that the exposure of microalgae to these compounds leads to an acquired removal capacity for these specific chemicals.



Statistical analyses showed that the total removal of OMPs during steady state of continuous mode was directly affected by the wastewater type. Specifically, the soluble COD of wastewater, dry weight and carbon uptake rate of the biomass positively influenced the total removal of OMPs.

To conclude, wastewater characteristics, such as soluble COD, and microalgae nutrient and carbon uptake rate, play an important role in the removal of OMPs by microalgae-based technology. To achieve a more efficient removal of OMPs, more biomass is needed in the bioreactors, which can be achieved by changing operational conditions (hydraulic and sludge retention times).

# Acknowledgements

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# SUPPLEMENTARY MATERIAL TO CHAPTER 3

**Table S3.1** The internal standards and recoveries (%) of OMPs in SPE extraction.

| OMPs | Internal standards | AnBW | MW | SCE |
|---|---|---|---|---|
| CAF | CAF-13c3 | 50 ± 3 | 86 ± 15 | 85 ± 9 |
| BTZ | BTZ-d4 | 54 ± 4 | 69 ± 8 | 79 ± 8 |
| SUL | SUL-d4 | 55 ± 4 | 93 ± 11 | 105 ± 11 |
| IBU | IBU-13c3 | 116 ± 15 | 51 ± 7 | 47 ± 3 |
| FUR | FUR-d5 | 40 ± 4 | 38 ± 5 | 78 ± 5 |
| PRO | PRO-d7 | 49 ± 5 | 88 ± 13 | 91 ± 5 |
| HYD | HYD-d2 | 56 ± 5 | 93 ± 11 | 85 ± 15 |
| MeBT | MeBT-d3 | 45 ± 1 | 89 ± 13 | 87 ± 7 |
| CLA | CLA-d3 | 67 ± 3 | 60 ± 2 | 99 ± 5 |
| TRI | TRI-d9 | 56 ± 4 | 87 ± 14 | 100 ± 5 |
| IRB | IRB-d4 | 54 ± 8 | 66 ± 2 | 96 ± 5 |
| MET | MET-d7 | 38 ± 5 | 82 ± 11 | 87 ± 6 |
| CBZ | CBZ-13c6 | 71 ± 7 | 43 ± 2 | 105 ± 3 |
| MCPA | MCPA-d3 | 88 ± 12 | 87 ± 2 | 104 ± 15 |
| MCPP | MCPP-d3 | 79 ± 7 | 86 ± 2 | 94 ± 9 |
| DCF | DCF-d4 | 99 ± 6 | 42 ± 2 | 92 ± 6 |

**Table S3.2** The standard deviations (%) of OMPs removal in all wastewater.

| OMPs | AnBW | | MW | | SCE | |
|---|---|---|---|---|---|---|
| | **Batch** | **Continuous** | **Batch** | **Continuous** | **Batch** | **Continuous** |
| CAF | 0 | 8 | 0 | 0 | 4 | 8 |
| BTZ | 1 | 7 | 7 | 5 | 2 | 4 |
| SUL | 2 | 7 | 15 | 5 | 4 | 2 |
| IBU | 0 | 9 | 2 | 6 | 3 | 6 |
| FUR | 4 | 4 | 7 | 7 | 0 | 4 |
| PRO | 3 | 9 | 8 | 7 | 4 | 4 |
| HYD | 0 | 3 | 5 | 5 | 1 | 3 |
| MeBT | 7 | 5 | 0 | 6 | 2 | 4 |
| CLA | 6 | 11 | 9 | 7 | 5 | 7 |
| TRI | 3 | 3 | 0 | 6 | 1 | 5 |
| IRB | 2 | 3 | 5 | 5 | 8 | 6 |
| MET | 4 | 4 | 0 | 3 | 1 | 4 |
| CBZ | 2 | 3 | 1 | 6 | 1 | 3 |
| MCPP | 6 | 4 | 6 | 4 | 1 | 2 |
| MCPA | 2 | 3 | 6 | 4 | 1 | 4 |
| DCF | 1 | 5 | 4 | 4 | 1 | 3 |

**Table S3.3** Nutrient (C/N/P) molar uptake rate (mmol/ (l. d)) of biomass in all wastewater during steady state.

| Wastewater | C | N | P |
|---|---|---|---|
| AnBW | 44.5 ± 5.6 | 6.5 ± 0.8 | 0.2 ± 0.03 |
| MW | 74.8 ± 0.3 | 6.2 ± 0.02 | 0.3 ± 0 |
| SCE | 3.5 ± 0.02 | 0.1 ± 0 | 0.01 ± 0 |

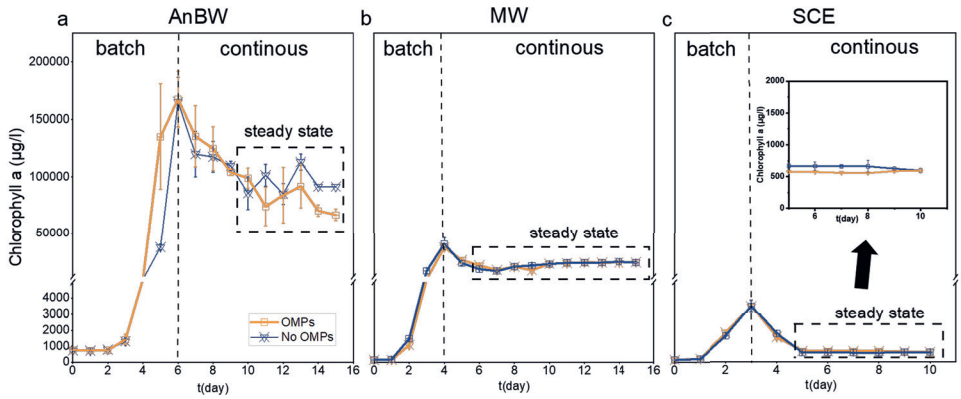



**Figure S3.1** Chlorophyll a of *Chlorella sorokiniana* in time in AnBW (a), MW (b) and SCE (c).



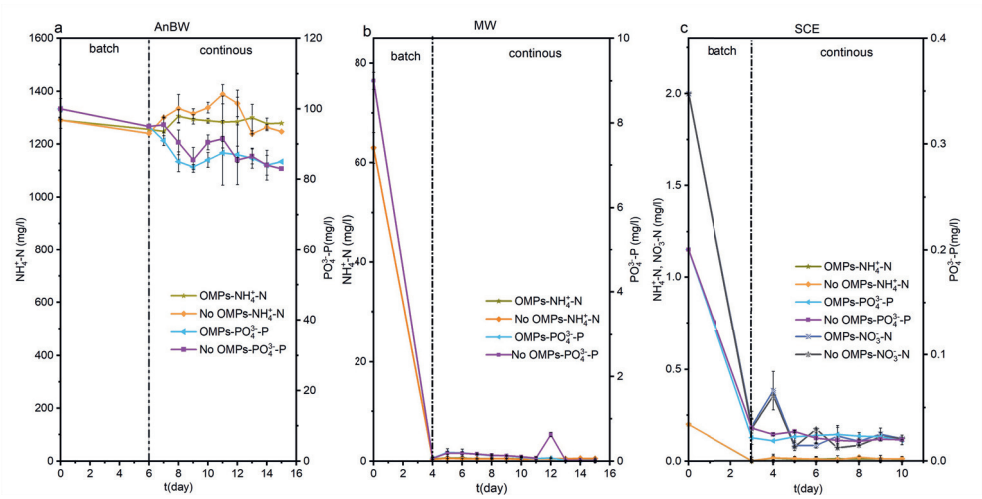



**Figure S3.2** Nutrient removal in time in AnBW (a), MW (b) and SCE (c).

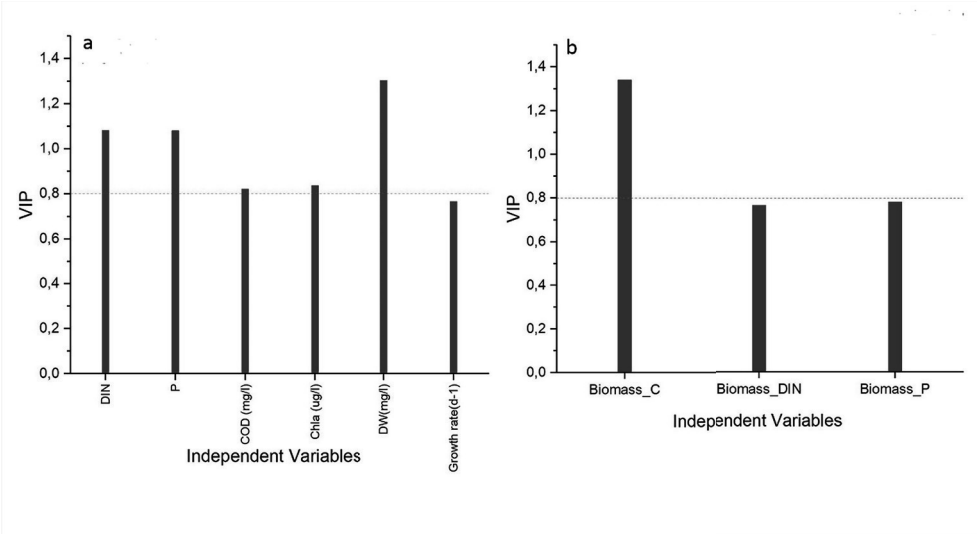
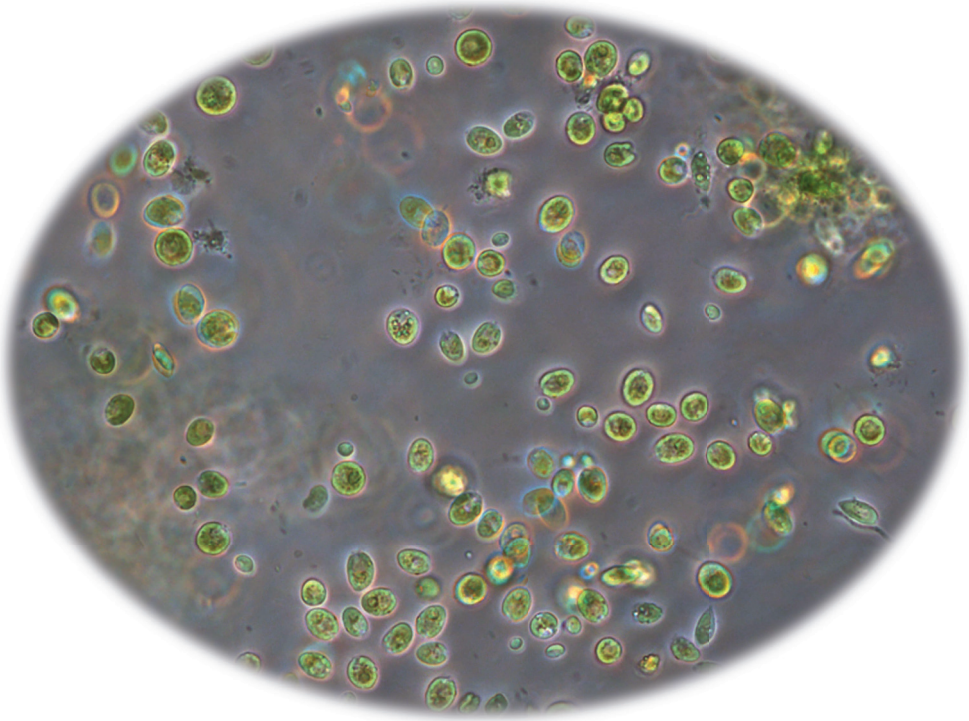



**Figure S3.3** Variable importance plot for wastewater characteristics and kinetic parameters (a), nutrient uptake of the biomass (b) on the total removal of OMPs.



# Chapter 4

# Impact of mixed microalgal species and bacteria on the removal of organic micropollutants in photobioreactors under natural light

Kaiyi Wu, Tino Leliveld, Hans Zweers, Huub Rijnaarts, Alette Langenhoff, Tânia V. Fernandes

# Abstract

Increased microalgae species diversity might increase the organic micropollutants (OMPs) removal from wastewater. In this study, two 27.5L photobioreactors were operated under continuous mode (HRT: 2 days) for 112 days to assess the removal efficiencies of 16 OMPs under spring/summer Dutch light conditions. One photobioreactor was inoculated with *Chlorella sorokiniana* and the other with a mixed microalgal-bacterial community. Three cultivation media were used in sequence; synthetic sewage for the first 28 days (Period I), 10 times diluted anaerobically digested black water (AnBW) from day 28 to 94 (Period II) and 5 times diluted AnBW from day 94 (Period III). Twelve out of 16 OMPs were removed >30% in both photobioreactors. Removal efficiencies of more than 90% were found in both reactors for caffeine, sulfamethoxazole, and furosemide. For the other nine degradable OMPs (ibuprofen, clarithromycin, propranolol, benzotriazole, 4/5-methylbenzotriazole, hydrochlorothiazide, metoprolol, diclofenac, and irbesartan), the *Chlorella sorokiniana* photobioreactor showed 30 to 95% removal efficiencies, which were similar or slightly higher than the mixed community photobioreactor. Furthermore, the *Chlorella sorokiniana* photobioreactor showed higher removal capacities of these nine compounds per biomass during the steady state of Period II than Period III, possibly due to more microalgae in Period II. The mixed community photobioreactor also had higher removal capacities during the steady state of Period II than Period III, possibly due to the invasion of filamentous green algae from the influent in Period III. Whereas the removal efficiencies were comparable, the mixed community photobioreactor showed higher removal capacities during the steady state of Period II. This indicates that the positive interactions between microalgal and bacterial species increased the removal capacities of OMPs. Hence, increasing biodiversity can be an efficient strategy for optimising the microalgae-based OMPs removal processes in full-scale wastewater treatment.

**Keywords:** microalgae, OMPs, photodegradation kinetics, biodegradation kinetics, bioaccumulation

# 4.1 Introduction

Organic micropollutants (OMPs) are present in both influent and effluent of conventional wastewater treatment plants (WWTP) from ng/l to µg/l (Yang et al., 2014). They can disrupt the endocrine systems of aquatic organisms, thereby negatively affecting ecosystems (Santos et al., 2007; Yang et al., 2014). So far, many wastewater treatment technologies, such as ozonation or activated carbon, have been shown to efficiently remove a wide range of OMPs from wastewater, but they require a high energy demand and material costs (Margot et al., 2013; Wei et al., 2017). Alternatively, microalgae-based technology is a sustainable biological treatment that can efficiently remove OMPs from wastewater, while recovering carbon and nutrients in algal biomass (Hena et al., 2021; Song et al., 2022).

Single algae species, cultivated in varying medium compositions and conditions, have shown to be efficient at OMPs removal (Nguyen et al., 2020; Liu et al., 2021; Song et al., 2022; Wu et al., 2022; Wu et al., 2023). Furthermore, it was recently shown that cultivating mixed microalgal communities alone or together with bacterial communities can enhance OMPs removal, because the positive cooperations between microalgal and bacterial species can result in higher removal efficiencies of OMPs (Hena et al., 2021). Katam et al. (2020) found that cultivating a mixed algal-bacterial culture, obtained from a 40 cm deep pond, removed > 96% of caffeine and linear alkylbenzene sulphonate from synthetic sewage in trickling filters under continuous mode. Batch incubations with a mixed algal-bacterial community dominated by *Chlollera sp.* and *Scenedesmus sp.* removed >90% of caffeine, ibuprofen, diclofenac and triclosan from urban wastewater (Matamoros et al., 2016). Prosenc et al. (2021) used lab-scale batch experiments to study mixed algal-bacterial biomass, obtained from a high rate algal pond, and found a 32% higher removal efficiency of bisphenols when compared to *C. vulgaris*. A mixed algal community, consisting of *Scenedesmus obliquus*, *Chlamydomonas mexicana*, *Chlorella vulgaris*, *Ourococcus multisporus*, and *Micractinium resseri*, showed a similar or higher removal efficiency of enrofloxacin than the individual species (Xiong et al., 2017a).

In this study, biomass concentration, nutrient removal and community composition were monitored in two photobioreactors inoculated with *Chlorella sorokiniana* and a mixed community of green algae, cyanobacteria, and hetero-chemotrophic bacteria under Dutch natural light conditions in spring/summer. Furthermore, the removal of 16 OMPs in these photobioreactors was investigated to elucidate the impact of species richness on OMPs removal.

# 4.2 Materials and methods

## 4.2.1 Cultivation medium

Synthetic sewage and 10 and 5 times diluted AnBW were used in this study as cultivation media (Table 4.1). Synthetic sewage was used for the cultivation of microalgae in batch mode until a high biomass density was reached, and consecutively used during the first 28 days (Period I) of the continuous mode. Diluted AnBW (10x) was used as feeding medium from day 28 to 94 (Period II) and changed to 5 times diluted from day 94 to112 (Period III). Tap water from a 1 $m^3$ tank was used for diluting AnBW.

**Table 4.1** Average characteristics (± standard deviation) of the used media.

| | **Synthetic sewage (n = 3)** | **Diluted AnBW (10x) (n = 8)** | **Diluted AnBW (5x) (n = 4)** |
|---|---|---|---|
| $COD_{soluble}$ (mg/l) | 712 ± 5 | 99 ± 10 | 201 ± 4 |
| TN (mg/l) | 230 ± 4 | 89 ± 4 | 175 ± 6 |
| $NH_4^+$-N (mg/l) | 66 ± 2 | 83 ± 6 | 172 ± 2 |
| TP (mg/l) | 13 ± 1 | 8 ± 0 | 15 ± 2 |
| $PO_4^{3-}$-P (mg/l) | 8 ± 0 | 7 ± 1 | 13 ± 1 |
| N/P molar ratio | 17/1 | 25/1 | 29/1 |

Synthetic sewage was modified from OECD method 303 (OECD, 2001), to mimic the soluble COD and nutrient concentrations of the wastewater of Bennekom, The

Netherlands (Table S4.1). Prior to use, synthetic sewage was autoclaved at 121 °C for 90 minutes and stored at 4 °C under anaerobic conditions.

AnBW was collected from the black-water treating upflow anaerobic sludge blanket (UASB) reactor of the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, The Netherlands. Fifty litres of AnBW were collected every twenty days. During the collection, AnBW was filtered through a 3 mm iron sieve to remove the undigested seeds, and therefore avoiding clogging of the photobioreactor tubing. Afterwards, AnBW was autoclaved at 121 °C for 90 minutes and stored at 4 °C under anaerobic conditions.

### 4.2.2 Microalgae and bacterial species

Five green microalgal species (*Chlorella sorokiniana*, *Scenedesmus obliquus*, *Chlorococcum sp.*, *Chlamydomonas reinhardtii* and *Haematococcus pluvialis*), and one cyanobacterium species (*Synechocystis sp.*) were chosen based on our previous experience, availability in the culture collection at NIOO-KNAW, and applicability to wastewater treatment (Bhatt et al., 2022). These species were maintained at 24 °C under continuous average incident irradiation of 80 μmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 16/8 h). *Chlorella sorokiniana*, *Scenedesmus obliquus*, *Chlorococcum sp.*, and *Chlamydomonas reinhardtii*, were cultivated in M8a medium (Kliphuis et al., 2010), while *Haematococcus pluvialis* and *Synechocystis sp.* were cultivated in WC medium (Kilham et al., 1998).

Activated sludge was collected from the Bennekom WWTP and used as source of mixed bacteria in this study. In this WWTP, municipal wastewater is treated by a conventional activated sludge system, a settling tank and a sand filtration (Lei et al., 2023).

### 4.2.3 Target OMPs

The target OMPs are caffeine (CAF), sulfamethoxazole (SUL), furosemide (FUR), ibuprofen (IBU), clarithromycin (CLA), propranolol (PRO), benzotriazole (BTZ), 4/5-methylbenzotriazole (MeBT), metoprolol (MET), hydrochlorothiazide (HYD), diclofenac (DCF), irbesartan (IRB), trimethoprim (TRI), mecoprop (MCPP), 2-

methyl-4-chlorophenoxyacetic acid (MCPA) and carbamazepine (CBZ). An OMPs stock of 60 µg/l per compound in tap water was prepared as previously described (Wu et al., 2022), and stored at 4 °C to maintain constant OMPs concentrations. This OMPs stock solution was added to the synthetic sewage or diluted AnBW to a final concentration of 6 µg/l, which was used as influent to the photobioreactors. Since caffeine was originally present in AnBW, its concentration in diluted AnBW (10x) and diluted AnBW (5x) was much higher, respectively 30 ± 1 and 62 ± 1 µg/l.

### 4.2.4 Experimental set-up

Two 27.5L bubble column photobioreactors were operated in a temperature-controlled greenhouse (25 °C) at NIOO-KNAW under natural light conditions from April to August 2022 (Figure 4.1). The *Chlorella sorokiniana* photobioreactor was inoculated at 16.9 µg chlorophyll/l. The mixed community photobioreactor was inoculated with the same total chlorophyll of all six algal species (equal to 1.6 mg dry weight/l) at equal proportions and 1.6 mg dry weight/l of activated sludge. The pH of both photobioreactors was automatically controlled at 6.8 ± 0.1 by acid (1M HCl) and base (1 M NaOH). Each photobioreactor was aerated with 5% $CO_2$/air at a flow rate of 1.1 l/min to homogeneously mix the content and supply $CO_2$ for photoautotrophic growth.

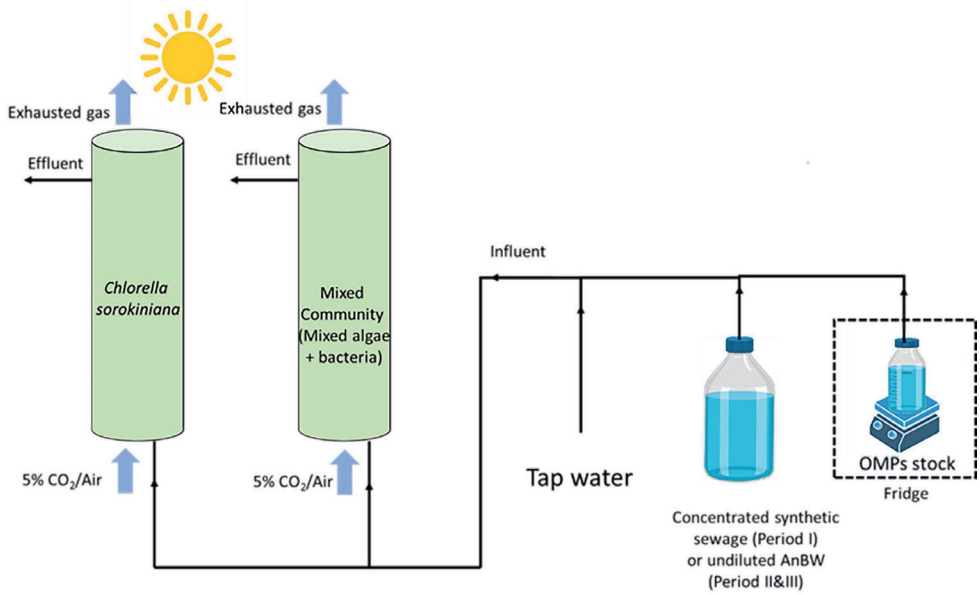



**Figure 4.1** Schematic representation of the experimental set-up.

The photobioreactors were initially operated in batch mode until the microalgal growth decreased due to P limitation. Thereafter, continuous mode (HRT: 2 days) was applied for 112 days.

## 4.2.5 Analytical methods

Dry weight and inorganic nutrients ($NH_4^+$-N, $NO_3^-$-N, $NO_2^-$-N, and $PO_4^{3-}$-P) in both photobioreactors were measured daily as previously described (Wu et al., 2022). After day 80, chlorophyll a was extracted daily from the biomass in 0.5 ml effluent samples and measured by a high-performance liquid chromatography (HPLC, UltiMate 3000; Thermo Scientific, USA). The details of extraction and analysis are described in Jin et al. (2022).

Microscopic observations were performed twice a week by using an inverted microscope (DMI3000B, Leica Microsystems Ltd., Germany) with a maximum magnification of 40x.

OMPs were measured in the undiluted AnBW, OMPs stock, effluent, and the biomass. Five ml of undiluted AnBW was collected weekly, and 5 ml of effluent samples were collected daily from both photobioreactors. Afterwards, these samples were centrifuged at 4500 rpm for 10 min. Additionally, five ml of OMPs stock was sampled weekly and diluted 10 times with demi water. Three ml of supernatant and diluted OMPs stock were used for extraction of OMPs by solid phase extraction (SPE) as described in Wu et al. (2022). The SPE recoveries of the used OMPs ranged from 47 ± 3 to 97 ± 10% (Table S4.2). Biomass was collected after centrifuging (4500 rpm, 10 min) 100 ml of effluent samples from both photobioreactors at six time points. The OMPs in the biomass were extracted by Quechers method, as previously described (Wu et al., 2022). The Quechers recoveries of the used OMPs ranged from 45 ± 1 to 104 ± 3% (Table S4.3) and were reproducible.

Both extracts were used to detect the OMPs by a liquid chromatograph coupled to a triple quadruple mass spectrometer (LC-MSMS), as described in Wu et al. (2023).

## 4.2.5 Calculations

Light intensity was measured daily by a GRAD radiation pyrometer that is placed on the roof of the greenhouse. The J/cm² data were converted into photo flux density (PFD) as described in Boelee et al. (2012).

Steady state was defined as a time slot when dry weight and chlorophyll a are stable (horizontal trendline), with a maximum standard deviation of 20% for > 5 consecutive days.

The OMP removal efficiency refers to the ratio between the removed OMP concentration ($\Delta c$; µg/l) and OMP influent concentration ($c_{inf}$; µg/l) (equation 4.2). The OMP removal capacity refers to the ratio between the removed OMP concentration and biomass concentration (dry weight (dw; g/l)) (equation 4.3). The removal capacities of CAF, SUL, and FUR were not calculated because their removal efficiencies were saturated (mostly >95%) in both photobioreactors (Figure 4.3). Such a calculation would lead to an underestimation of the actual removal capacities of these OMPs. TRI, MCPP, MCPA, and CBZ were also excluded due to their negligible removal efficiencies in both photobioreactors (Figure S4.1).

$$\Delta c = c_{inf} - c_t \tag{4.1}$$

$$OMP\ removal\ efficiency\ (\%) = \frac{\Delta c}{c_{inf}} \times 100\% \tag{4.2}$$

$$OMP\ removal\ capacity\ (\mu g/g\ dw) = \frac{\Delta c}{dw} \tag{4.3}$$

where
$\Delta c$ (µg/l) = removed OMP concentration;
$c_{inf}$ (µg/l) = OMP influent concentration;
$c_t$ (µg/l) = OMP concentration at sampling time;
dw (mg/l) = dry weight at sampling time.

# 4.3 Results and discussion

## 4.3.1 Growth dynamics

Biomass productivity, assessed by dry weight and chlorophyll a, followed a similar trend in both photobioreactors (Figure 4.2a, b), except for the beginning of Period III in the *Chlorella sorokiniana* photobioreactor (day 97 to 103), where dry weight did not increase as sharply as chlorophyll a. This difference was most likely due to contamination and subsequence overgrowth of filamentous green algae in the photobioreactor with mixed community from the tap water container (Figure S4.2) at the end of period II (around day 80). Even though the tap water was fed into both photobioreactors, the overgrowth of filamentous green algae in the *Chlorella sorokiniana* photobioreactor did not take place as there was no available $PO_4^{3-}$-P at the end of period II (Figure 4.2c). In the mixed community photobioreactor, $PO_4^{3-}$-P was available at the end of period II (Figure 4.2d). Together with the doubling concentrations of $NH_4^+$-N from period III onwards, the filamentous green algae invader had the perfect conditions to grow. It has been shown that filamentous green algae can outcompete other microalgae, due to its higher surface area for photo adsorption (Liu et al., 2020). This filamentous green algal growth ceased when nitrifiers grew exponentially in period III, shown by the increase in the concentrations of $NO_3^-$-N and $PO_4^{3-}$-P (Figure 4.2c, d). Nitrifiers do not use $PO_4^{3-}$-P but $NH_4^+$-N for growth. When conditions are optimal, such as sufficient shading from the microalgal culture, they can outcompete the microalgae. Previous studies showed that such lower light conditions (in this case sufficient shading) favoured the growth of nitrifiers, including ammonia oxidizing bacteria and nitrite oxidizing bacteria (Guerrero and Jones, 1997; Vergara et al., 2016).

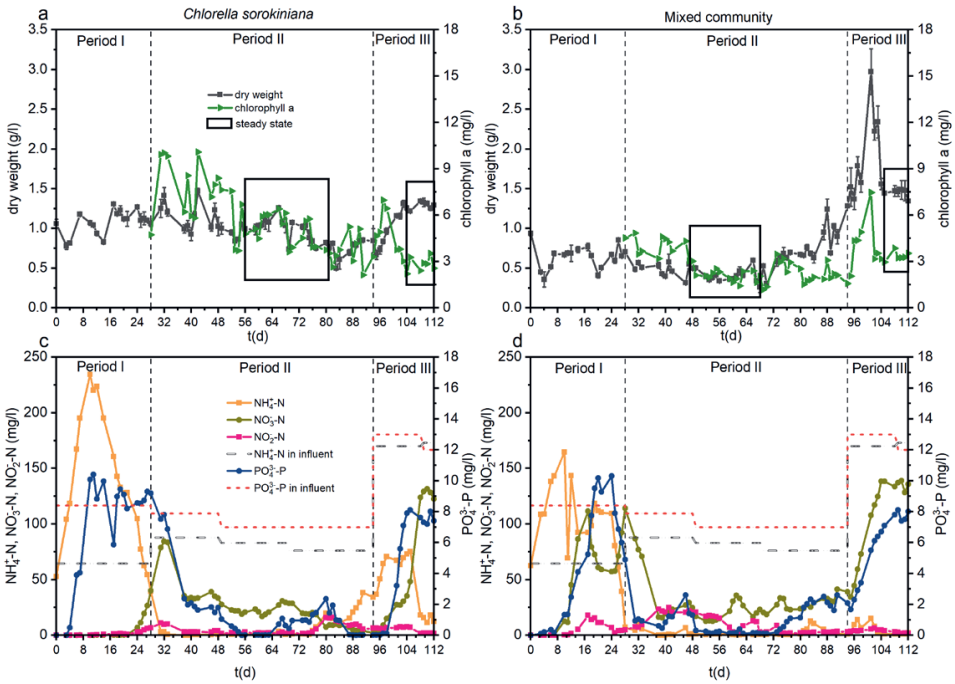



**Figure 4.2** Dry weight and chlorophyll a, in the photobioreactors inoculated with *Chlorella sorokiniana* (a) and a mixed community (b). $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$-N, and $PO_4^{3-}$-P concentrations in the photobioreactors inoculated with *Chlorella sorokiniana* (c) and a mixed community (d). Medium $NH_4^+$-N and $PO_4^{3-}$-P concentrations are presented as dashed lines. Period I: synthetic sewage; Period II: 10x diluted AnBW, and Period III: 5x diluted AnBW.

When evaluating the performance of the photobioreactors, the dry weight in the *Chlorella sorokiniana* photobioreactor (1.1 ± 0.2 g/l) was almost double the dry weight in the mixed community photobioreactor (0.6 ± 0.1 g/l) in Period I. The nitrifiers in the mixed community photobioreactor started to grow from day 10, while they only grew from day 24 in the *Chlorella sorokiniana* photobioreactor, which can be seen by the increase in $NO_3^-$-N concentration (Figure 4.2c, d). This was because the inoculated activated sludge used in the mixed community photobioreactor already contained nitrifiers. Additionally, an increase in $NH_4^+$-N and $PO_4^{3-}$-P concentrations was observed in both photobioreactors (Figure 4.2c, d), sometimes

higher than the medium concentrations. Heterotrophic bacteria were likely present and converted the organic TN and TP in the medium (peptone and meat extract) to their inorganic form ($NH_4^+$-N and $PO_4^{3-}$-P).

In period II, a pseudo-steady state was selected per photobioreactor based on a 5 days period with a constant dry weight and chlorophyll a concentrations, with < 20% standard deviation. The pseudo-steady state of the *Chlorella sorokiniana* photobioreactor was from day 56 to 80, while the one for the mixed community photobioreactor was from day 49 to 63. During the steady state, dry weight and chlorophyll a in the *Chlorella sorokiniana* photobioreactor were more than two-fold higher than those of the mixed community photobioreactor, respectively 1.0 ± 0.1 g dry weight/l and 4.9 ± 1.0 mg chlorophyll a/l, and 0.4 ± 0.0 g dry weight/l and 2.0 ± 0.3 mg chlorophyll a/l (Figure 4.2a, b). This difference might have been due to the lack of competition for nutrients in the *Chlorella sorokiniana* photobioreactor in relation to the mixed community photobioreactor. Even though *Chlorella sorokiniana* is a fast-growing species when cultivated in nutrient-rich environments, it grows slower when cultivated in a mixed community (Corcoran et al., 2019; Schmidt et al., 2020). Similarly, *Chlorella sp.*, *Scenedesmus obliquus*, and *Spirulina platensis* showed higher biomass productivity than the mixed community, when cultivated in open tanks on municipal wastewater in another study (Bhattacharjee and Siemann, 2015). Cross-contamination was not observed in the tanks with individual species. Schmidtke et al. (2010) and Thomas et al. (2019) reported that species richness (0 to 8 species) did not increase the biomass concentration, since the species richness in their studies was not sufficient to enhance the resource complementary utilization by microalgae. This can also explain the lower biomass concentration in mixed community in our study.

Nitrifiers grew similarly for both photobioreactors, with identical concentration of $NO_3^-$-N of 22.3 ± 8.3 mg/l and 22.7 ± 4.6 mg/l for the *Chlorella sorokiniana* and the mixed community photobioreactors, respectively.

In Period III, the dry weight and chlorophyll a during pseudo-steady states (day 104 to 112) were similar in both photobioreactors. Dry weight and chlorophyll a were 1.3 ± 0.1 g dry weight/l and 2.9 ± 0.5 mg chlorophyll a/l in the *Chlorella sorokiniana* photobioreactor, and 1.5 ± 0.1 g dry weight/l and 3.3 ± 0.3 mg chlorophyll a/l for the

mixed community photobioreactor (Figure 4.2a, b). The high $NO_3^-$-N concentration of 124.0 ± 2.5 mg/l (*Chlorella sorokiniana*) and 135.7 ± 3.7 mg/l (mixed community) at the end of Period III for both reactors, together with the high $PO_4^{3-}$-P concentration, indicate that the microalgae were not optimally growing (Figure 4.2c, d). This might be a result of the time needed for microalgae to adapt to the change in N source, from $NH_4^+$-N to $NO_3^-$-N, which is metabolically more energy consuming (Maestrini et al., 1986; Bhaya et al., 2002; Sanz-Luque et al., 2015). A longer operation of the photobioreactors would possibly result in a new increase in the microalgae community biomass, and therefore a decrease in the $NO_3^-$-N, and $PO_4^{3-}$-P effluent concentrations.

## 4.3.2 OMPs removal

Twelve out of sixteen OMPs were removed in both photobioreactors (Figure 4.3). In addition, the contribution of biosorption (the sum of bioadsorption and bioaccumulation) was negligible (<3%) to the removal efficiencies of the tested OMPs (Table S4.4), which is in line with previous studies (De Wilt et al., 2016; Wu et al., 2022). Therefore, photodegradation and biodegradation were the main removal processes for these OMPs in this study. TRI, MCPP, MCPA, and CBZ were not removed (Figure S4.1), and the recalcitrance of these four OMPs is in line with our previous studies with AnBW and artificial medium (De Wilt et al., 2016; Wu et al., 2022, 2023).

CAF, SUL, and FUR were removed for more than 90% in both photobioreactors (Figure 4.3a, b, c). These high removal efficiencies were also shown in other pilot- and lab-scale photobioreactors (Matamoros et al., 2015; Hom-Diaz et al., 2017; Nguyen et al., 2020; García-Galán et al., 2020a; Prosenc et al., 2021). However, in our previous study, only 79% of SUL and 38% of FUR removal efficiency was observed under continuous mode (HRT: 0.8 days) in lab-scale photobioreactors with *Chlorella sorokiniana* in AnBW, although higher biomass concentration (1.7 g dry weight/l) was detected (Wu et al., 2023). Possibly, the higher HRT applied in the current study enhanced the interaction between the microorganisms and OMPs, thus increasing their removal efficiencies, as stated elsewhere (Matamoros and Rodríguez, 2016).

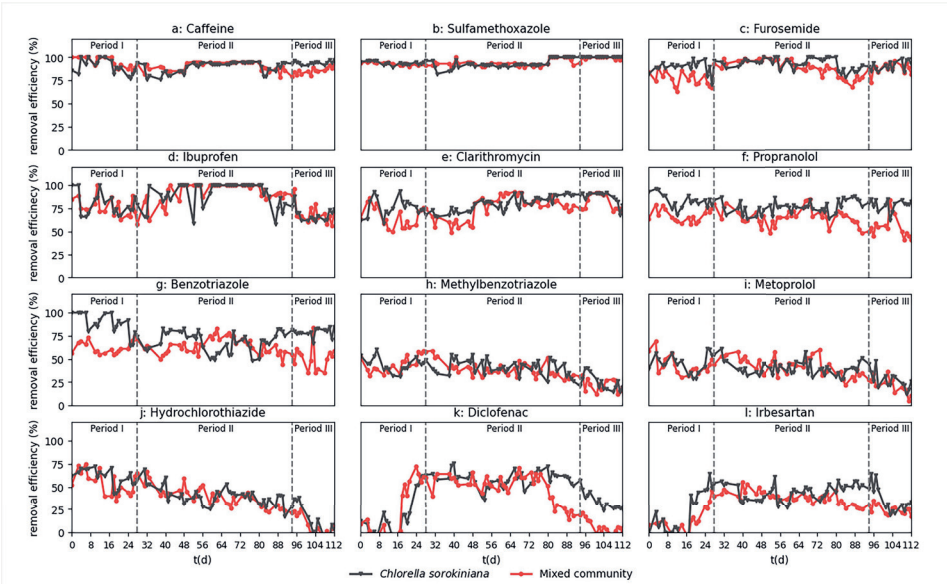



**Figure 4.3** Removal efficiencies (%) of 12 OMPs over time in photobioreactors with *Chlorella sorokiniana* and a mixed community. Period I: synthetic sewage; Period II: 10x diluted AnBW and Period III: 5x diluted AnBW.

IBU removal efficiency fluctuated from 75 to 95% in Period I and II in both photobioreactors and decreased to 66 ± 5% removal efficiency in Period III (Figure 4.3d). CLA, PRO, and BTZ removal efficiency ranged from 50 to 95% in all three periods in the *Chlorella sorokiniana* photobioreactor, and was slightly lower in the mixed community photobioreactor (Figure 4.3e, f, g).

Removal efficiencies of MeBT, MET, HYD, DCF, and IRB ranged from 30 to 70% in both photobioreactors with especially low removal efficiencies during period III (Figure 4.3h, i, j, k, l). The only exception was DCF, where a higher removal efficiency was observed in the *Chlorella sorokiniana* photobioreactor after 80 days, compared to the mixed community photobioreactor. A negative removal efficiency was observed for HYD, DCF and IRB. Most likely, these fluctuations and even negative removal efficiencies were due to the reversible conjunction and deconjunction processes between OMPs metabolites, which was also seen in other biological systems (Plósz et al., 2012; Vieno and Sillanpää, 2014; Nguyen, 2021).

Except for BTZ, the removal capacities in the *Chlorella sorokiniana* photobioreactor for these nine OMPs were higher during the steady state of Period II than Period III (Figure 4.4). This coincides with the decrease of chlorophyll a (Figure 4.2a), showing that less microalgae were present in Period III. This indicates that microalgae might be responsible for the removal of these OMPs in the *Chlorella sorokiniana* photobioreactor. As BTZ showed similar removal capacity in both steady states, other microorganisms than microalgae contributed to BTZ removal in this photobioreactor, e.g. bacteria, such as nitrifiers (Herzog et al., 2014; Wagner et al., 2020; Wang et al., 2022).

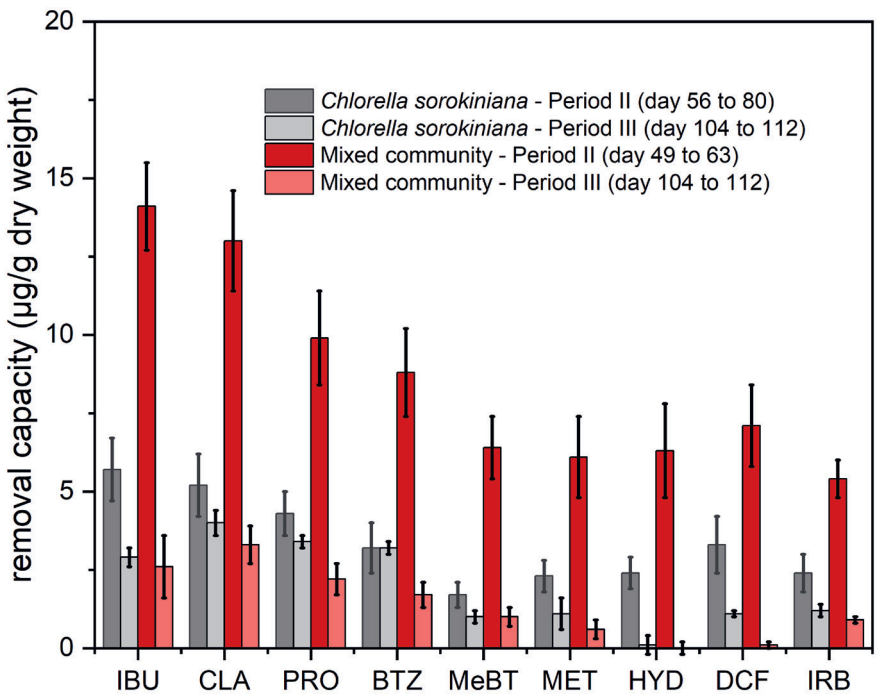



**Figure 4.4** Removal capacities (µg/ g dry weight) of 9 OMPs during steady states of continuous mode in the photobioreactors with *Chlorella sorokiniana* and a mixed community.

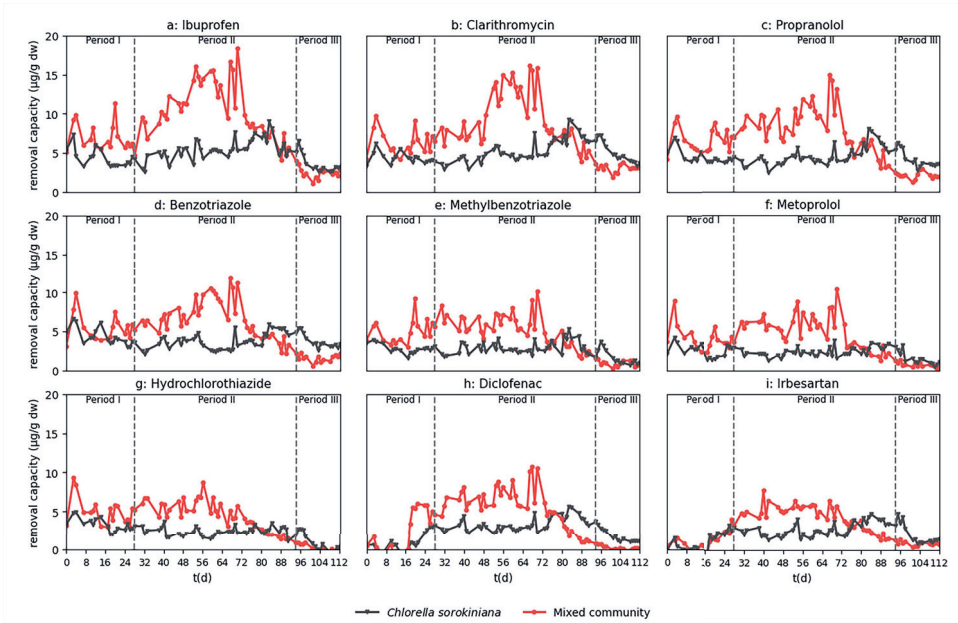



**Figure 4.5** Removal capacities (µg/ g dry weight) over time of 9 OMPs in the photobioreactors with *Chlorella sorokiniana* and a mixed community. Period I: synthetic sewage; Period II: 10x diluted AnBW; Period III: 5x diluted AnBW.

A similar observation was seen in the removal capacities in the mixed community photobioreactor; higher during the steady state of Period II than Period III.

When comparing the two photobioreactors, the mixed community photobioreactor showed higher removal capacities from day 0 to 80 (Figure 4.5), including the steady state of Period II. It is known that cooperative interactions between microalgal and bacterial species can improve the removal capacities of some OMPs (Hena et al., 2021). Also Xiao et al. (2021) showed a higher removal of cefradine with a mixed microalgal community of *C. pyrenoidosa* and *M. aeruginosa* than with the individual species. They hypothesized that the allelochemicals, produced in the mixed microalgal community of *C. pyrenoidosa* and *M. aeruginosa*, influenced the metabolism and enzymes of the microorganisms, thereby enhancing the removal of cefradine. Another study showed that co-cultivating activated sludge with green

algae or diatom removed linear alkylbenzene sulfonate better than green algae in a semi-batch photobioreactor with laundry wastewater (Pandey et al., 2020).

However, the opposite was seen after day 80, when more biomass was present in the mixed community photobioreactor (Figure 4.5). The predominance of filamentous green algae in Period III are mostly likely responsible for the decrease in the OMPs removal capacities in the mixed community photobioreactor.

Thus, to further optimise the removal efficiencies of degradable OMPs, microalgal and bacterial communities should be co-cultivated. Additionally, it can be beneficial to retain more biomass in the photobioreactor with mixed community by engineering measures, such as decoupling hydraulic retention time from solid retention time.

## 4.4 Conclusion



Three out of sixteen OMPs (CAF, SUL, and FUR) were completely removed in both photobioreactors, while CBZ, MCPP, MCPA and TRI were poorly removed (<10 %). The removal efficiencies of the other nine OMPs varied from 30 to 95%, and the *Chlorella sorokiniana* photobioreactor showed similar, or slightly higher removal efficiencies than the mixed community photobioreactor.

The *Chlorella sorokiniana* photobioreactor showed higher removal capacities of these nine OMPs during the steady state of Period II than III, most likely due to the decreased microalgae concentration. In the mixed community photobioreactor, lower OMPs removal capacities were found during the steady state of Period III, possibly due to the predominance of filamentous green algae from tap water. Furthermore, the mixed community photobioreactor exhibited higher OMPs removal capacities than *Chlorella sorokiniana* during the steady state of Period II, indicating the positive impact of adding more microalgal species and bacteria.

# Acknowledgements

This research was financially supported by STOWA, the Dutch Foundation for Applied Water Research (Amersfoort, The Netherlands), OASEN drinking water company (Gouda, The Netherlands), Waterboard De Dommel (Bosscheweg, The Netherlands) and Waterboard Vallei en Veluwe (Apeldoorn, The Netherlands). The authors also want to thank Livio Carlucci and Beatriz Alvarado Perry for their assistance on ibuprofen and furosemide measurement. The financial support from Guangzhou Elite Project (GEP) for the research and study of Kaiyi Wu is kindly acknowledged.

# SUPPLEMENTARY MATERIAL TO CHAPTER 4

**Table S4.1** Characteristics of influent in Bennekom, The Netherlands.

| | | Average | Median | Max value | Min value | Standard deviation | Perc. 95% | Number of measurements |
|---|---|---|---|---|---|---|---|---|
| COD | mg/l | 712 | 720 | 1320 | 140 | 209 | 1030 | 278 |
| BOD5 | mg/l | 272 | 280 | 470 | 49.0 | 84.3 | 410 | 277 |
| NKj | mg/l | 64.7 | 69.7 | 95.7 | 11.3 | 18.9 | 87.8 | 278 |
| NH4 | mg/l | 64.5 | 64.5 | 64.5 | 64.5 | 0 | 64.5 | 1 |
| Ntot | mg/l | 64.7 | 69.7 | 95.7 | 11.3 | 18.9 | 87.8 | 278 |
| Ptot | mg/l | 8.52 | 8.90 | 13.0 | 1.60 | 2.49 | 12.0 | 279 |
| OB | mg/l | 273 | 260 | 820 | 52.0 | 110 | 472 | 277 |
| Cl | mg/l | 67.3 | 69.0 | 110 | 13.0 | 28.9 | 103 | 6 |
| TZV | mg/l | 1007 | 1036 | 1724 | 192 | 280 | 1380 | 278 |
| Cd | ug/l | 0.20 | 0.19 | 0.28 | 0.14 | 0.048 | 0.28 | 10 |
| Cr | ug/l | 4.33 | 3.90 | 7.40 | 2.60 | 1.43 | 6.95 | 10 |
| Cu | ug/l | 53.1 | 52.0 | 77.0 | 38.0 | 11.1 | 70.7 | 10 |
| Pb | ug/l | 9.37 | 6.85 | 21.0 | 4.20 | 5.22 | 19.2 | 10 |
| Ni | ug/l | 4.01 | 3.90 | 5.70 | 3.10 | 0.79 | 5.39 | 10 |
| Zn | ug/l | 192 | 175 | 310 | 130 | 52,5 | 274 | 10 |
| Hg | ug/l | 0.12 | 0.080 | 0.48 | 0.020 | 0.13 | 0.34 | 10 |
| As | ug/l | 1.73 | 1.70 | 2.50 | 1.00 | 0.48 | 2.41 | 10 |
| Fe | mg/l | 0.74 | 0.59 | 2.00 | 0.32 | 0.48 | 1.64 | 10 |
| Al | ug/l | 892 | 825 | 1500 | 480 | 331 | 1500 | 10 |
| Sb | ug/l | 0.78 | 0.60 | 1.60 | 0.60 | 0.32 | 1.38 | 10 |
| V | ug/l | 1.65 | 1.10 | 4.10 | 1.00 | 1.07 | 3.79 | 10 |
| Ag | ug/l | 0.92 | 1.00 | 1.00 | 0.23 | 0.23 | 1.00 | 10 |
| Ba | ug/l | 33.1 | 32.5 | 50.0 | 20.0 | 9.30 | 48.2 | 10 |
| Be | ug/l | 0.051 | 0.050 | 0.060 | 0.050 | 0.003 | 0.056 | 10 |
| Ca | mg/l | 53.1 | 53.5 | 61.0 | 44.0 | 5.34 | 60.6 | 10 |
| Co | ug/l | 0.51 | 0.45 | 0.91 | 0.29 | 0.20 | 0.87 | 10 |
| K | mg/l | 26.7 | 26.5 | 31.0 | 24.0 | 1.95 | 30.1 | 10 |
| Mg | mg/l | 6.58 | 6.70 | 7.30 | 5.80 | 0.48 | 7.21 | 10 |
| Mn | mg/l | 0.11 | 0.094 | 0.17 | 0.059 | 0.035 | 0.17 | 10 |
| Mo | ug/l | 2.21 | 2.05 | 3.30 | 1.60 | 0.46 | 2.99 | 10 |
| Na | mg/l | 62.9 | 63.5 | 74.0 | 50.0 | 6.22 | 71.3 | 10 |
| Sn | ug/l | 4.38 | 4.40 | 5.90 | 2.80 | 1.11 | 5.90 | 10 |
| Sr | ug/l | 153 | 150 | 180 | 120 | 18.5 | 176 | 10 |
| Te | ug/l | 0.21 | 0.20 | 0.25 | 0.20 | 0.015 | 0.23 | 10 |
| Tl | ug/l | 0.46 | 0.50 | 0.50 | 0.050 | 0.14 | 0.50 | 10 |
| U | ug/l | 0.31 | 0.23 | 0.70 | 0.19 | 0.18 | 0.61 | 6 |
| W | ug/l | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 1.00 | 3 |

**Table S4.2** SPE recoveries (%) of OMPs in this study**.**

| OMPs | Internal standards | Synthetic sewage | AnBW (5x) | AnBW (10x) | Undiluted AnBW | Diluted OMPs stock |
|---|---|---|---|---|---|---|
| CAF | CAF-13D3 | 89 ± 7 | 97 ± 10 | 91 ± 0 | 93 ± 3 | 87 ± 6 |
| BTZ | BTZ-D4 | 77 ± 3 | 70 ± 0 | 75 ± 2 | 78 ± 2 | 90 ± 1 |
| SUL | SUL-D4 | 55 ± 4 | 93 ± 11 | 105 ± 11 | 67 ± 3 | 95 ± 2 |
| IBU | IBU-13C3 | 89 ± 6 | 55 ± 4 | 47 ± 3 | 78 ± 1 | 89 ± 2 |
| FUR | FUR-D5 | 63 ± 7 | 61 ± 7 | 73 ± 6 | 81 ± 3 | 93 ± 3 |
| PRO | PRO-D7 | 62 ± 5 | 59 ± 3 | 80 ± 5 | 73 ± 1 | 85 ± 3 |
| HYD | HYD-D2 | 79 ± 5 | 78 ± 9 | 85 ± 6 | 88 ± 2 | 95 ± 1 |
| MeBT | MeBT-D3 | 55 ± 6 | 61 ± 8 | 87 ± 7 | 77 ± 3 | 88 ± 3 |
| CLA | CLA-D3 | 62 ± 7 | 58 ± 5 | 59 ± 5 | 64 ± 2 | 79 ± 1 |
| TRI | TRI-D9 | 84 ± 3 | 85 ± 0 | 84 ± 9 | 84 ± 5 | 85 ± 3 |
| IRB | IRB-D4 | 75 ± 4 | 69 ± 3 | 71 ± 3 | 78 ± 4 | 91 ± 5 |
| MET | MET-D7 | 55 ± 5 | 89 ± 7 | 75 ± 2 | 60 ± 3 | 99 ± 5 |
| CBZ | CBZ-13C6 | 88 ± 2 | 87 ± 0 | 85 ± 8 | 88 ± 8 | 89 ± 3 |
| MCPA | MCPA-D3 | 94 ± 3 | 87 ± 2 | 80 ± 4 | 81 ± 3 | 83 ± 2 |
| MCPP | MCPP-D3 | 77 ± 1 | 76 ± 5 | 69 ± 3 | 83 ± 4 | 89 ± 3 |
| DCF | DCF-D4 | 88 ± 3 | 85 ± 5 | 88 ± 1 | 78 ± 5 | 93 ± 2 |

**Table S4.3** Quechers recoveries (%) of OMPs in different media in this study.

| OMPs | Internal standards | *Chlorella sorokiniana* | Mixed community |
|---|---|---|---|
| CAF | CAF-13C3 | 103 ± 10 | 67 ± 6 |
| BTZ | BTZ-D4 | 86 ± 3 | 74 ± 1 |
| SUL | SUL-D4 | 62 ± 2 | 56 ± 3 |
| IBU | IBU-13C3 | 86 ± 1 | 72 ± 3 |
| FUR | FUR-D5 | 51 ± 0 | 100 ± 2 |
| PRO | PRO-D7 | 98 ± 5 | 95 ± 3 |
| HYD | HYD-D2 | 118 ± 7 | 82 ± 2 |
| MeBT | MeBT-D3 | 97 ± 2 | 81 ± 5 |
| CLA | CLA-D3 | 81 ± 2 | 104 ± 3 |
| TRI | TRI-D9 | 53 ± 2 | 61 ± 7 |
| IRB | IRB-D4 | 69 ± 4 | 90 ± 3 |
| MET | MET-D7 | 73 ± 1 | 78 ± 7 |
| CBZ | CBZ-13C6 | 48 ± 2 | 80 ± 4 |
| MCPA | MCPA-D3 | 66 ± 4 | 55 ± 1 |
| MCPP | MCPP-D3 | 56 ± 2 | 75 ± 3 |
| DCF | DCF-D4 | 66 ± 4 | 68 ± 3 |

**Table S4.4** Biosorption (%) of 16 OMPs in both photobioreactors.

| OMPs | *Chlorella sorokiniana* | Mixed community |
|---|---|---|
| CAF | 1.0 ± 0.3 | 1.3 ± 0.6 |
| BTZ | 1.0 ± 0.5 | 1.7 ± 0.3 |
| SUL | 0.9 ± 0.1 | 1.2 ± 0.2 |
| IBU | 0.9 ± 0.2 | 1.3 ± 0.1 |
| FUR | 1.0 ± 0.0 | 1.2 ± 0.3 |
| PRO | 0.9 ± 0.1 | 1.3 ± 0.0 |
| HYD | 0.6 ± 0.0 | 0.6 ± 0.0 |
| MeBT | 0.1 ± 0.3 | 0.4 ± 0.0 |
| CLA | 2.1 ± 0.2 | 3.4 ± 0.1 |
| TRI | 0.0 ± 0.0 | 2.1 ± 0.2 |
| IRB | 0.0 ± 0.0 | 1.3 ± 0.5 |
| MET | 1.8 ± 0.1 | 1.2 ± 0.5 |
| CBZ | 1.1 ± 0.2 | 1.3 ± 0.4 |
| MCPA | 0.5 ± 0.1 | 0.6 ± 0.2 |
| MCPP | 0.0 ± 0.0 | 0.4 ± 0.0 |
| DCF | 0.5 ± 0.2 | 3.6 ± 0.3 |

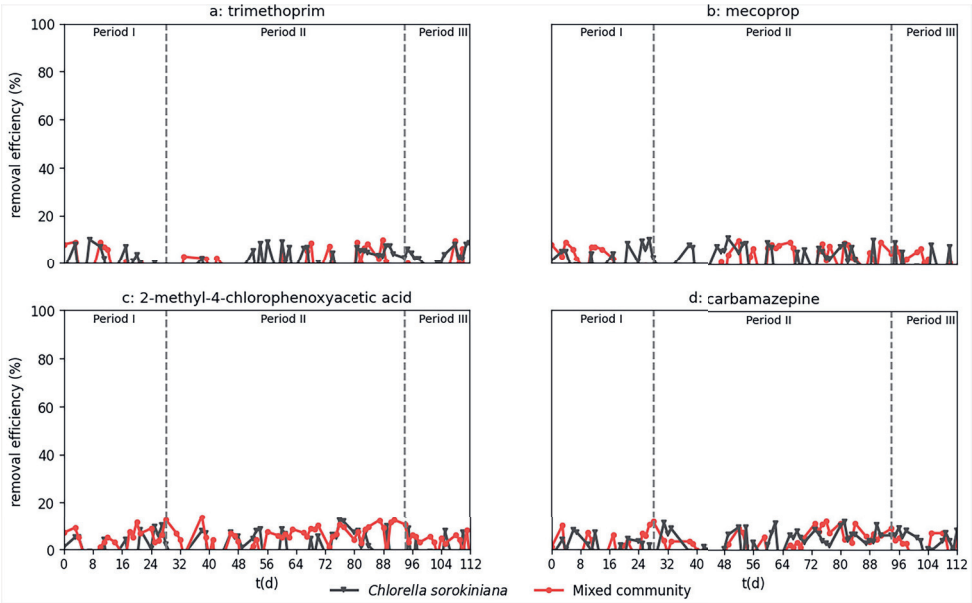



**Figure S4.1** Removal efficiencies (%) of 4 OMPs over time in the photobioreactors with *Chlorella sorokiniana* and a mixed community. Period I: synthetic sewage; Period II: 10x diluted AnBW and Period III: 5x diluted AnBW.

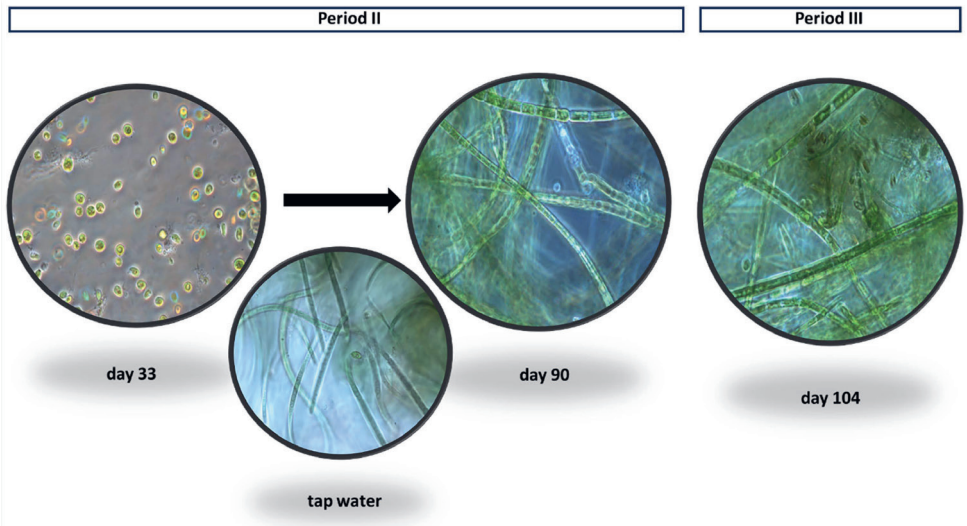



**Figure S4.2** Microscopic images (magnification: 400x) of microorganisms in the photobioreactors with a mixed community in Period II and III and tap water.

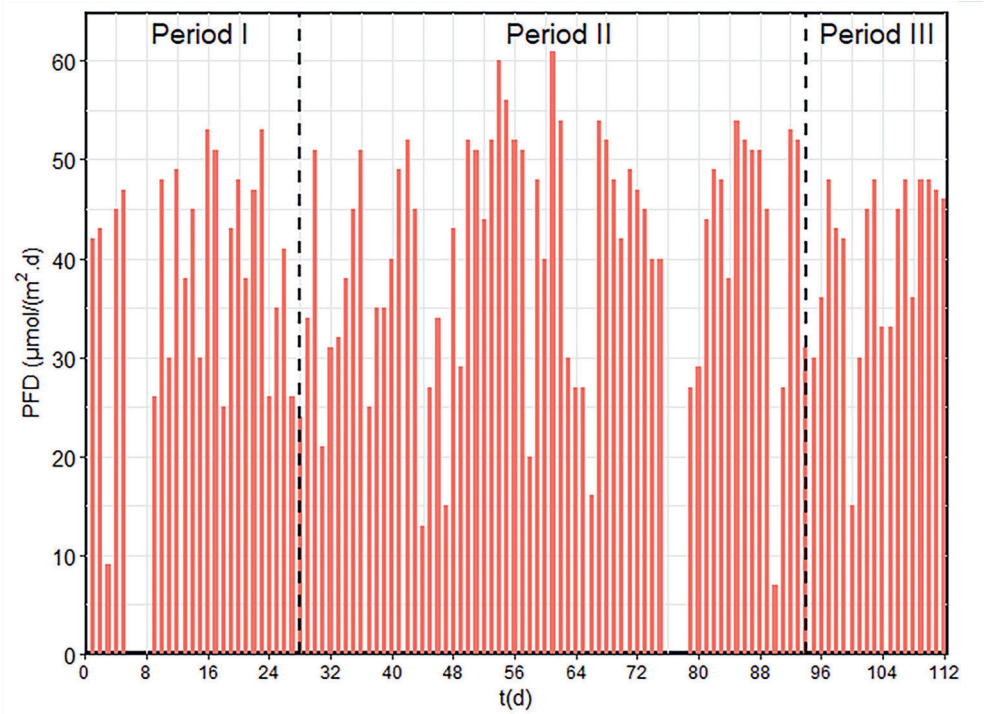
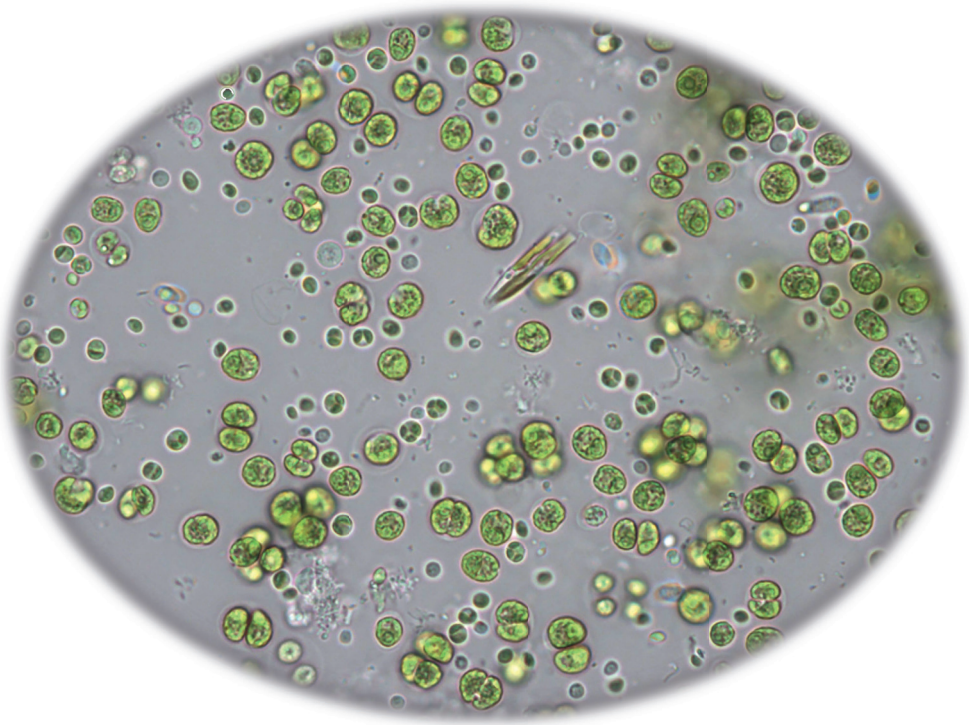



**Figure S4.3** Photo flux density (PFD) of sunlight at the roof of the greenhouse throughout the experiment.



# Chapter 5

# Effect of species richness on the removal of organic micropollutants in microalgae-based systems

Kaiyi Wu, Raphaël Vercoustre, Gloria Martínez Pereira, Hans Zweers,

Huub Rijnaarts, Alette Langenhoff, Tânia V. Fernandes

# Abstract

Higher species richness in a microalgal community appears to be important for designing an efficient microalgae-based organic micropollutants (OMPs) removal process. In this study, we conducted flask and chemostat experiments (HRT: 5 days) to investigate the impact of microalgal and bacterial species richness on OMPs removal efficiency. The flask experiments with green algae, cyanobacteria and chemoheterotrophic bacteria were conducted in batch mode for 8 days to avoid light limitation. However, carbon and P limitation was observed during the experiments. In the chemostat experiments, diatoms were added to the previously tested taxa. The chemostats were first operated in batch mode followed by continuous mode. In the flask experiments, microalgal species richness effect on removal efficiency was absent for caffeine (CAF), carbamazepine (CBZ) sulfamethoxazole (SUL), metoprolol (MET), and benzotriazole (BTZ). However, for 2-methyl-4-chlorophenoxyacetic acid (MCPA), hydrochlorothiazide (HYD), ibuprofen (IBU) and diclofenac (DCF), microalgal species richness increased the removal efficiency with 17 to 20%. Furthermore, adding bacteria caused a 5 to 29% increase in MET, MCPA, HYD, and DCF removal efficiency, and >50% for BTZ and IBU. In the chemostat experiments, species richness effect was similar in batch mode and continuous mode, except for clarithromycin (CLA). No effect was seen in SUL and MET removal efficiency. For BTZ, DCF, and MCPA, a 20 to 49% increase was seen when microalgal species richness increased. For CLA, this positive effect was only seen in continuous mode. Furthermore, a slight negative or no effect of adding bacteria was seen for all OMPs.

The effect of microalgal species richness and bacteria could not be demonstrated for most OMPs in this study.

**Keywords:** compounds of emerging concerns, biodiversity taxa, phytoplankton, biodegradation

# 5.1 Introduction

Wastewater treatment and reuse is important for addressing the global issue of water quality and scarcity, arising from urbanization and population growth (Singhal and Perez-Garcia, 2016; Meena et al., 2019). A crucial step is to efficiently remove organic micropollutants (OMPs) from wastewater. OMPs can negatively affect the metabolism of aquatic organisms, such as fish and snails, and thereby ecosystems, even though they are generally present from ng/l to µg/l (Tufi et al., 2016; Muter and Bartkevics, 2020). Microalgae-based technology has proven to be a sustainable technology for efficiently removing many OMPs from wastewater (Tolboom et al., 2019; Usmani et al., 2020).

Applying suitable microalgal species is key for designing microalgae-based OMPs removal technologies (Hena et al., 2021; Liu et al., 2021). Furthermore, studies have shown that communities with higher microalgal and bacterial species richness enhanced the removal of OMPs, such as bisphenol, efradine, and norfloxacin (Stravs et al., 2017, 2019; Hena et al., 2021; Prosenc et al., 2021; Xiao et al., 2021). This can be attributed to the positive microalgal interspecies and microalgal-bacterial interactions (Hena et al., 2021; Xiao et al., 2021). Therefore, co-cultivating multiple microalgal species with varying nutrient utilization capacities, including N:P uptake ratio, can result in higher complementarity in nutrient use and therefore higher phytoplankton biomass (Fernandes et al., 2017; Gonçalves et al., 2017; Padmaperuma et al., 2018). Adding bacteria to a microalgal community can also increase biomass production by complementarity in resource acquisition, such as exchange of metabolites, e.g. vitamin $B_{12}$ and dissolved organic compounds (Yao et al., 2019; Oviedo et al., 2022). Higher biomass generates more enzymes and adsorption sites, available for OMPs removal (Gonçalves et al., 2017; Oviedo et al., 2022). Furthermore, as different types of microalgae and bacteria have various enzymes responsible for OMPs degradation, these enzymes can contribute to the different OMPs degradation, thereby reaching an efficient removal (Thomas and Hand, 2012; Stravs et al., 2019; Massot et al., 2022). Also, adding bacteria can improve the removal efficiency of the present OMPs in the system, as bacteria are able to remove for example norfloxacin and ketoprofen, whereas microalgae were

not as effective (Ismail et al., 2016; Xiao et al., 2021). For example, a mixed community of *Chlorella pyrenoidosa* and *Microcystis aeruginosa* showed a higher removal efficiency of cefradine than the individual species in the flask experiments, and co-cultivating these species with bacteria from activated sludge removed 58% of norfloxacin after 24 h (Xiao et al., 2021).

Many studies focused on green microalgae, and seldomly use cyanobacteria or diatoms, which are also efficient in removing OMPs (Ding et al., 2019; Fang et al., 2023; Wang et al., 2020; Zhang et al., 2023). For example, Fang et al. (2023) reported that 33 to 66% of sulfamethoxazole and ofloxacin (both antibiotics) were removed by the cyanobacteria *Synechococcus sp.* and *Chroococcus sp.* after 4 days of batch incubation. The diatom *Skeletonema costatum* removed 67% of dicofol, 76% of acetochlor and 77% of chlorpyrifos after 14 days of batch incubation (Zhang et al., 2023). Thus, studies on microalgal species richness, including taxonomic diversity, are needed for elucidating how biodiversity affected microalgae-based OMPs removal.

Furthermore, most studies are performed in flasks in batch mode under controlled conditions, and seldomly conducted at continuous mode, which is generally applied in wastewater treatment plants (WWTP) (Grandclément et al., 2017). Operational mode (batch or continuous) can affect OMPs removal by influencing the contact time between OMPs and biomass, and the amount of biomass available for OMPs removal (Matamoros and Rodríguez, 2016; Hena et al., 2021; Wu et al., 2023).

In this study, four microalgal species (3GA and 1CY) and bacteria from activated sludge were used to investigate the impact of microalgal and bacterial species richness on biomass growth and removal efficiencies of 9 OMPs in batch mode in flasks. Further, 11 microalgal species (4GA, 3CY, and 3DT) and bacteria from activated sludge were tested for the removal of 6 compounds in chemostats operated in batch and continuous mode (HRT: 5 days).

# 5.2 Materials and methods

## 5.2.1 Cultivation medium

The media applied in flask experiment and chemostat experiment (Table S5.1) were modified from BG-11 medium (Bold, 1949). In short, acetate was added as carbon source in the flask experiments, while meat extract and peptone were added as carbon source in the chemostat experiments, as well as $Na_2SiO_3.3H_2O$ as silicon source for diatom growth.

## 5.2.2 Microalgal and bacterial species

Four green algae species, two cyanobacteria species, and two diatom species (Table 5.1) originated from the culture collection of NIOO-KNAW, the Netherlands. Green algal species and cyanobacteria species were individually maintained at BG-11 medium at 24 °C under continuous average incident irradiation of 80 μmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 16/8 h), whereas diatom species were individually maintained at WC medium (Kilham et al., 1998) at 24 °C under continuous average irradiation of 70 μmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 24 °C). *Chroococcus sp.* and *Nitzschia palea* were acquired from Belgian culture collections (BCCM, Belgium). Afterwards, they were individually transferred to BG-11 medium at the same temperature and incident irradiation as green algal species and cyanobacteria species from NIOO-KNAW.



**Table 5.1** Microalgal species used in this study.

| Green algae (GA) | Cyanobacteria (CY) | Diatoms (DT) |
|---|---|---|
| *Chlorella sorokiniana* | *Synechocystis sp.* | *Nitzschia palea* |
| *Scenedesmus obliquus* | *Synechococcus elongtus* | *Cyclotella meneghiniana* |
| *Chlorococcum sp.* | *Chroococcus sp.* | *Diatoma cf. tenuis* |
| *Chlamydomonas reinhardtii* | | |

In the flask experiments, *Chlorococcum sp., Chlamydomonas reinhardtii, Scenedesmus obliquus,* and *Synechocystis sp.* were adapted to the sterilized medium

without OMPs until their grow rates were constant for minimal 7 generations. The adaptation was conducted in 1L sterilized Erlenmeyer flasks in a temperature-controlled climate room (21 ± 1 °C) with fluorescent lights in the ceiling. The light intensity was adjustable allowing for an average intensity of 107 ± 18 μmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 12/12 h). The flasks were daily randomised to achieve identical light exposure. Each flask was bubbled with 33 ml/min of air enriched with 5% $CO_2$.

Activated sludge was used as a source of chemoheterotrophic bacteria. It was collected from the biological treatment process of the wastewater treatment plant (WWTP) in Bennekom, The Netherlands. The collected activated sludge was immediately used and adapted to our experimental conditions (medium and temperature) in the flask experiments in the dark. For the chemostat experiments, the activated sludge was stored at 4 °C overnight, prior to its use.

## 5.2.3 Target OMPs

The target OMPs for the flask experiments were caffeine (CAF), sulfamethoxazole (SUL), metoprolol (MET), benzotriazole (BTZ), diclofenac (DCF), 2-methyl-4-chlorophenoxyacetic acid (MCPA), hydrochlorothiazide (HYD), ibuprofen (IBU), and carbamazepine (CBZ). The concentration of each OMP was 150 μg/l in the medium.

The target OMPs for the chemostat experiments were sulfamethoxazole (SUL), metoprolol (MET), benzotriazole (BTZ), diclofenac (DCF), and 2-methyl-4-chlorophenoxyacetic acid (MCPA), and clarithromycin (CLA). The concentration of each OMP was 10 μg/l in the medium.

OMPs were spiked into the media as previously described (Wu et al., 2022).

## 5.2.4 Flask experimental set-up

The experiments with microalgae (1GA + 1CY and 3GA + 1CY) and microalgae and bacteria (3GA + 1CY + BAC) treatments (Table 5.2) were conducted under the same conditions as the adaptation phase (see 5.2.2). All treatments were conducted in triplicate. All flasks were inoculated with a total chlorophyll of 11.5 ± 0.4 μg/l, at equal proportion of each microalgal species, into 600 ml of sterilized medium (with

OMPs). In the 3GA + 1CY + BAC treatment, the activated sludge was inoculated at the same dry weight as the added microalgae ($6.8 \times 10^{-4}$ mg dry weight/l). Abiotic control was applied to investigate the contribution of OMPs photodegradation. Biotic control without spiking OMPs, was applied to identify the effect of OMPs on the growth of microalgae and bacteria. All treatments and controls were incubated for 8 days to avoid light limitation.

**Table 5.2** Microalgal species inoculated in the treatments and biotic controls in the flask experiments.

| 1GA + 1CY | 3GA + 1CY | 3GA + 1CY + BAC |
|---|---|---|
| *Chlorococcum sp.* (GA) | *Chlorococcum sp.* (GA) | *Chlorococcum sp.* (GA) |
| *Synechocystis sp.* (CY) | *Chlamydomonas reinhardtii* (GA) | *Chlamydomonas reinhardtii* (GA) |
| | *Scenedesmus obliquus* (GA) | *Scenedesmus obliquus* (GA) |
| | *Synechocystis sp.* (CY) | *Synechocystis sp.* (CY) |



## 5.2.5 Chemostat experimental set-up

The chemostat experiments were conducted in four 380 ml chemostats (Wu et al., 2023). One chemostat was inoculated with all four green algal species (4GA), two chemostats were inoculated with all ten microalgal species (4GA + 3CY + 3DT), and one chemostat was inoculated with all ten microalgal species plus activated sludge (4GA + 3CY + 3DT + BAC) at the equal dry weight as total microalgae dry weight (Table 5.3). The inoculated concentrations of microalgae were the same as for the flask experiments. Temperature (21 ± 1 °C) and pH (6.8 ± 0.1) were automatically controlled. The content in each chemostat was mixed by bubbling 10% $CO_2$ enriched air at a total flow rate of 100 ml/min. A sinus curve with a maximum average light intensity of 100 µmol $m^{-2}$ $s^{-1}$ (light/dark cycle: 16/8 h) was applied for the light regime.

**Table 5.3** Microalgal species inoculated in the chemostat experiments.

| **4GA** | **4GA + 3CY + 3DT** | **4GA + 3CY + 3DT + BAC** |
|---|---|---|
| *Chlorella sorokiniana* (GA) | *Chlorella sorokiniana* (GA) | *Chlorella sorokiniana* (GA) |
| *Scenedesmus obliquus* (GA) | *Scenedesmus obliquus* (GA) | *Scenedesmus obliquus* (GA) |
| *Chlorococcum sp.* (GA) | *Chlorococcum sp.* (GA) | *Chlorococcum sp.* (GA) |
| *Chlamydomonas reinhardtii* (GA) | *Chlamydomonas reinhardtii* (GA) | *Chlamydomona reinhardtii* (GA) |
| | *Synechocystis sp.* (CY) | *Synechocystis sp.* (CY) |
| | *Synechococcus elongtus* (CY) | *Synechococcus elongtus* (CY) |
| | *Chroococcus sp.* (CY) | *Chroococcus sp.* (CY) |
| | *Nitzschia palea* (DT) | *Nitzschia palea* (DT) |
| | *Cyclotella meneghiniana* (DT) | *Cyclotella meneghiniana* (DT) |
| | *Diatoma cf. tenuis* (DT) | *Diatoma cf. tenuis* (DT) |

All chemostats were operated in batch mode until the end of the exponential growth phase of the algae and switched to continuous mode (HRT: 5 days) until steady state was observed, defined as the time at which the dry weight, total chlorophyll, and chlorophyll a were constant for a minimum of five consecutive days, with a standard deviation lower than 5%.

## 5.2.6 Analytic methods

In the flask experiments, biomass concentration was determined in duplicate by dry weight at day 8, according to a standard method (Rice and American Public Health Association, 2012). Chemical oxygen demand (COD) was daily measured by a high-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, US), after filtering 1 ml of algal samples through 0.22 μm polyethersulfone syringe filters (VWR, The Netherlands).

In the chemostat experiments, biomass concentration was quantified daily by dry weight, total chlorophyll, and chlorophyll a during the continuous mode. Total chlorophyll was measured using a Phyto PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany), and chlorophyll a was extracted from biomass in 0.5 ml of samples by 80% ethanol and measured using a HPLC, as described in Jin et al. (2022). Since the phycobilins in cyanobacteria and carotenoids in diatoms were included in the Phyto-Pam measurements, total chlorophyll is higher than chlorophyll a when cyanobacteria and diatoms were present. Phyto PAM measurement is an indirect fluorescence measurement, and its accuracy depends on the used reference, so this measurement was more likely to be interfered and less accurate for quantifying the chlorophyll in the mixed community than individual species. COD was daily measured by using the LCK1414 COD kits (Hach Lange, The Netherlands) after 0.22 μm polyethersulfone filtration of samples.

In both experiments, inorganic nutrients ($NH_4^+$-N, $NO_3^-$-N, $NO_2^-$-N, and $PO_4^{3-}$-P), and OMPs in the medium, were measured daily and quantified as previously described (Wu et al., 2022). Bioadsorbed and bioaccumulated OMPs in the biomass were quantified at the end of the experiments. The Quechers recoveries of the used OMPs ranged from 55 ± 3 to 96 ± 2% (Table S5.2) and were reproducible. Microscopic observations were performed daily by using an inverted microscope (DMI3000B, Leica Microsystems Ltd., Germany) with a maximum magnification of 40x.

## 5.2.7 Statistical analysis

In the flask experiments, a Wilcoxon test was performed to identify the significant differences in dry weight between the treatment (with OMPs) and control (without

OMPs) in the groups with different species richness, and between the treatments with different species richness, as previously described (Wu et al., 2022). A student t-test was applied to identify the significant differences between dry weight, total chlorophyll, and chlorophyll a in paired groups during the steady state of continuous mode in chemostat experiments.

# 5.3 Results and discussion

## 5.3.1 Microalgal growth

In the flask experiments, the dry weight in 1GA + 1CY treatment (with OMPs) was 0.67 ± 0.05 mg/l, which is significantly lower than its biotic control (Figure 5.1), indicating a possible inhibitory effect of OMPs on the microalgal growth. It is known that OMPs like MET and DCF can negatively affect the growth of microalgae by interrupting the photosynthetic systems and protein synthesis (Miazek and Brozek-Pluska, 2019; Harshkova et al., 2021). Dry weight was not affected by OMPs in the other two treatments, but were affected by phosphate and carbon limitation (Figure S5.1, 5.2). However, species richness could have enhanced the tolerance of microalgal biomass to OMPs. In a microcosm experiment with seven species (*Bacillaria sp.*, *Coscinodiscus sp.*, *Ditylum sp.*, *Guinardia sp.*, *Gyrosigma sp.*, *Odontella sp.* and *Thalassiosira sp.*), a less negative effect of atrazine (25 µg/l) on biomass production was observed at higher species richness (Steudel et al., 2012).

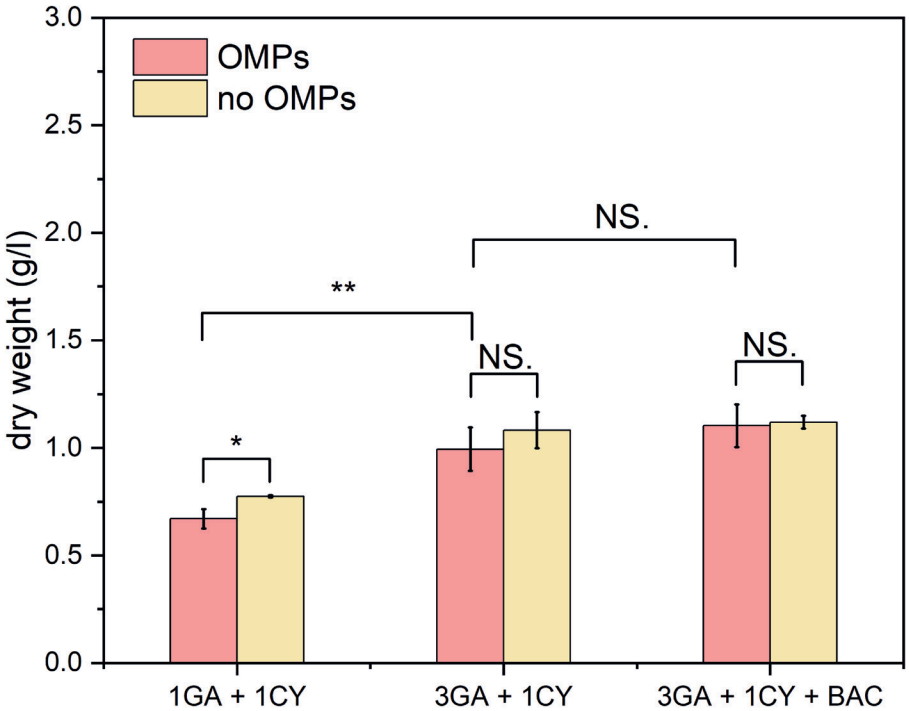



**Figure 5.1** Dry weight at day 8 of the flask experiments. Error bars represent standard deviations. NS. no significant difference, *P < 0.05, **P < 0.01, Wilcoxon test.

Treatment 3GA + 1CY showed a significantly higher dry weight (0.99 ± 0.13 g/l) than 1GA + 1CY, while there was no significant difference between 3GA + 1CY (0.99 ± 0.13 g/l) and 3GA + 1CY + BAC (1.10 ± 0.10 g/l) (Figure 5.1). Previous studies showed that multiple microalgal species can utilize the resources more efficiently than single species, therefore resulting in higher biomass (Corcoran and Boeing, 2012; Fouilland, 2012; Nath et al., 2017). However, in our experiments, both phosphate and carbon were limiting growth (Figure S5.1, 5.2), which might have affected heterotrophic microalgae and bacteria growth, and therefore dry weight.

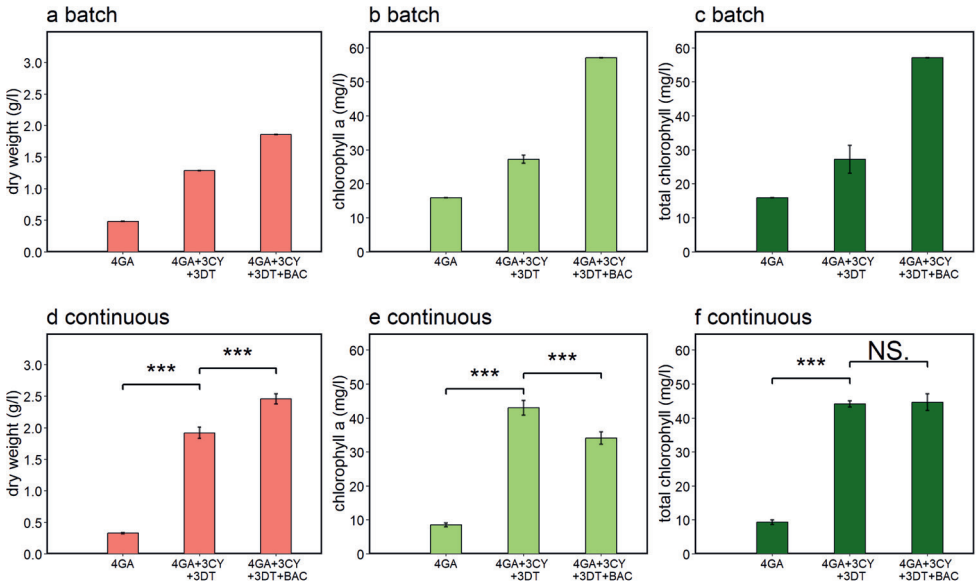



**Figure 5.2** Dry weight (a, d), total chlorophyll (b, e), and chlorophyll a (c, f) in batch and continuous mode in the chemostat experiments. In continuous mode, the numbers of data points (n) were respectively 7, 10, and 10 for 4GA, 4GA + 3CY + 3DT, and 4GA + 3CY + 3DT + BAC treatments. Error bars represent standard deviations. NS. no significant difference, ***P < 0.001, student's t-test.

In the chemostat experiments, the dry weight was 0.45 g/l in batch mode of 4GA treatment, which was lower than 3GA + 1CY treatment. A lower taxonomic richness in 4GA treatment possibly led to a less efficient utilization of resources, and thereby less biomass (Zhang and Zhang, 2006; Fouilland, 2012). Similarly with flask experiments, species richness significantly increased the biomass productivity in the chemostat experiments during batch mode, assessed by dry weight, total chlorophyll, and chlorophyll a (Figure 5.2). Additionally, when bacteria were added, a clear increase in biomass (76% for dry weight, 119% for total chlorophyll, and 118% for chlorophyll a) was seen in batch mode of the chemostat experiments (no carbon and P limitation). This supports our assumption that carbon limitation negatively affected microalgal and bacterial growth in the flask experiments. Moreover, adding bacteria enhances biomass production (Yao et al., 2019; Oviedo et al., 2022).

During the steady state of the continuous mode, a similar trend was found for the dry weight. The difference between 4GA and the other two treatments was however larger than during batch mode as the dry weight in GA treatment was underestimated. This was possibly due to an acid and base diluting incident, which also affected the chlorophyll a and total chlorophyll data (also underestimated). This upward trend was not shown in the total chlorophyll and chlorophyll a of the other two treatments. This can be explained by the different relative abundance of green algae, cyanobacteria, and diatoms of these two treatments, especially the varying amounts of diatoms, as indicated by the percentage of total chlorophyll of diatom (4% for 4GA + 3CY + 3DT, 11% for 4GA + 3CY + 3DT + BAC) and microscopic images (Figure S5.3). The cellular amount of total chlorophyll is microalgal species-dependent and can range from 0.1 to 9.7% (Hallegraeff, 1977; Boyer et al., 2009). Therefore, in the mixed microalgal communities, the changes in dry weight and chlorophyll can be different in various treatments with the same species (Ramaraj et al., 2013), as observed in our study.

### 5.3.2 OMPs removal efficiencies

In the flask experiment, six out of nine OMPs (CAF, SUL, DCF, MCPA, HYD, and IBU) were removed in the treatments with microalgae only. When bacteria were added, MET and BTZ were also removed, but CBZ remained recalcitrant.

In the chemostat experiments, all six added OMPs (SUL, MET, BTZ, DCF, MCPA, and CLA) were removed in all treatments. The removal efficiencies of SUL, MET and CLA were > 60% in both batch and continuous mode.

In both experiments, bioadsorption and bioaccumulation accounted for less than 5% removal for all OMPs (Table S5.3, S5.4), indicating the main removal processes were biodegradation for all OMPs and photodegradation only for DCF and IBU.

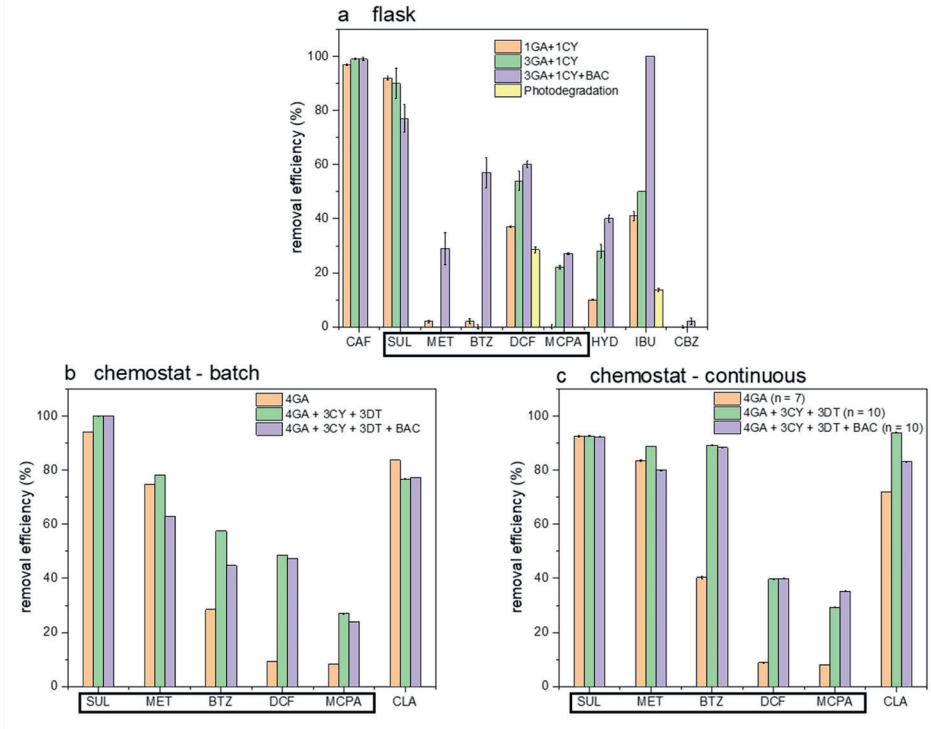



**Figure 5.3** OMPs removal efficiencies in flask and chemostat experiments. OMPs in boxes at the legend of the x-axis were tested in both experiments. Error bars represent standard deviations.

CAF was removed > 90% in all flask experiments (Figure 5.3). SUL was removed > 90% in the treatments with microalgae only. These high removal efficiencies of CAF and SUL are in line with previous studies (Gojkovic et al., 2019; Ding et al., 2020; Ferrando and Matamoros, 2020; Wu et al., 2023). When bacteria were added, only 77% SUL was removed. This treatment has similar dry weight with 3GA + 1CY treatment, indicating the presence of less microalgae. Therefore, a low SUL removal efficiency was achieved. On the other hand, SUL was removed >90% in both batch and continuous mode of the chemostat experiments. It seems that SUL removal efficiency was not affected by biomass concentration. Likely, the long batch duration (18 days) and continuous duration (30 days) has led to an optimal removal efficiency

for all treatments in the chemostat experiments compared to flask experiments with a duration of 8 days.

Negligible MET removal efficiency was observed in 1GA + 1CY and 3GA + 1CY treatments (flask experiments). In our previous flask experiments with single species (*Scenedesmus obliquus*), MET was also poorly removed (Wu et al., 2022). When bacteria were present, 29% of MET was removed, which can be explained by the contribution of bacteria, as MET can be used as carbon source by heterotrophic bacteria under carbon limitation (Hellauer et al., 2019). In the chemostat experiment, MET removal efficiency was 75% in batch mode of 4GA and 4GA + 3CY + 3 DT treatments, and >83% in the continuous mode. In comparison with the flask experiments (8 days), the longer batch incubation time (18 days) in the chemostat experiment resulted in an increased removal efficiency. However, in our previous experiments with *Chlorella sorokiniana*, we observed that MET was not removed for 15 days (Wu et al., 2023). It seems that certain microalgal species combinations could enhance MET removal efficiency. Xiao et al. (2021) proposed that the allelochemicals, released by *Chlorella pyrenoidosa* and *Microcystis aeruginosa* in a microalgal consortium, positively affected the metabolism and production of enzymes, and thereby the removal of OMPs, like cefradine. Furthermore, a 10% lower removal efficiency was seen in 4GA + 3CY + 3DT + BAC treatment compared to 4GA + 3CY + 3DT in both batch and continuous mode. The treatment of 4GA + 3CY + 3DT + BAC posed a higher proportion of diatom that are not as effective as green algae and cyanobacteria for removing OMPs. Similarly, the presence of diatom (*Asterionella Formosa*, *Fragilaria crotonensis*, *Nitzschia sp.*, *Synedra rumpens var. familiaris*, and *Tabellaria sp.*) in a mixed microalgal community (5 chlorophyta, 3 chrysophyta, 4 cryptophyta, and 5 cyanobacteria) negatively affected the degradation of cyprodinil, kresoxim methyl, fipronil, and fludioxonil, possibly due to diatom's low removal capacities of these compounds (Stravs et al., 2019).

BTZ was not removed in 1GA + 1CY and 3GA + 1CY treatments, but 57% of removal efficiency was observed in the presence of bacteria. In our previous experiments with single species in batch mode, 88% of removal efficiency was seen in anaerobically digested black water (AnBW) and municipal wastewater (MW), and no removal was observed in the batches with secondary clarified effluent (SCE), that

had a poor nutrient concentration (Wu et al., 2023). It seems that phosphate depletion limited the microalgal growth, thereby negatively affecting BTZ degradation in the flask experiments (batch mode). Additionally, heterotrophic bacteria contributed to BTZ removal under carbon limited conditions, which was also documented in previous studies (Müller et al., 2017; Wang et al., 2022). In the chemostat experiments, only 28% of BTZ was removed in batch mode of 4GA treatment. This lower removal efficiency compared to our previous study with *Chlorella sorokiniana* was likely due to the lower biomass concentration (0.45 g/l) in GA treatment. A remarkable increase was seen in 4GA + 3CY + 3DT treatment, indicating a possible positive effect of species richness. Similar with MET, the presence of bacteria resulted in 13% of decrease in BTZ removal efficiency, probably due to more diatom, which is less effective as green algae and cyanobacteria for BTZ removal. During the steady state of the chemostat continuous mode, BTZ removal efficiency was 40% in GA treatment. Furthermore, 89% of BTZ removal efficiency was observed in 4GA + 3CY + 3DT treatments with and without bacteria. For the treatment of 4GA, the long continuous duration (30 days) appeared to increase the capacity of BTZ removal, as has been previously reported (Hena et al., 2021; Wu et al., 2022), resulting in a higher removal during the steady state of continuous mode. For the other two treatments, the applied HRT (5 days) led to an increase of biomass, resulting in a higher removal efficiency.

DCF was photodegradable in the flask experiments, which was indicated by 28% removal efficiency in the photodegradation control. It was also seen in our previous studies, which were performed under similar fluorescent light (De Wilt et al., 2018; Wu et al., 2022). DCF removal efficiency was 37% in 1GA + 1CY treatment, and 17% higher in 3GA + 1CY treatment. The microalgal species richness increased the biomass concentration and thereby the DCF removal efficiency. When bacteria were added, DCF removal efficiency slightly increased to 60%. Only 9% of DCF was removed in batch mode of 4GA treatment. This lower removal efficiency can result from the absence of photodegradation compared to 3GA + 1CY treatment. DCF can only utilize the light with a wavelength < 350 nm (Rizzo et al., 2009; Salgado et al., 2013), which was not present in the light (400 to 800 nm) applied for chemostats. In our previous experiments with *Chlorella sorokiniana* in the same chemostats, DCF photodegradation was absent (Wu et al., 2023). However, DCF removal efficiency

in 4GA treatment remained lower than DCF biodegradation in 3GA + 1CY (26%), due to a lower biomass concentration. Furthermore, DCF removal efficiency in batch mode of 4GA + 3CY + 3DT treatment was 49%, which was higher than 4GA treatment. Adding bacteria did not further increase the DCF removal efficiency. During the steady state of continuous mode, DCF removal efficiency was similar or slightly lower than batch mode, and a similar positive effect of microalgal species richness and no effect of adding bacteria were observed.

MCPA was not removed in 1GA + 1CY treatment, which coincides with our previous study (Wu et al., 2023). The removal efficiency increased to 22% in 3GA + 1CY treatment, indicating the positive effect of increasing microalgal species richness. Adding bacteria slightly changed DCF removal to 27%, and a Wilcon test showed that this change was insignificant (results not shown). In the chemostat experiments, MCPA removal efficiency was only 8% in batch mode of 4GA treatment, which is lower than 3GA + 1CY treatment of flask experiments, again due to lower biomass concentration. MCPA was 27% removed from 4GA + 3CY + 3DT treatments with and without bacteria. This indicates the positive effect of microalgal species richness and limited effect of adding bacteria. During the steady state of continuous mode, the removal efficiency was similar.



HYD removal efficiency in 3GA + 1CY treatment was 28%, higher than 1GA + 1CY treatment (10%), and the presence of bacteria caused a 12% increase, indicating a positive effect of species richness on HYD removal efficiency. A similar removal efficiency (40%) was also seen in a high rate algal pond (HRT: 6 days) with urban wastewater (Villar-Navarro et al., 2018) and a 1200 L multitube photobioreactor (HRT: 4 days) with toilet water (Hom-Diaz et al., 2017).

IBU showed 14% photodegradation in the flask experiments, and a similar photodegradation was also seen in previous studies (De Wilt et al., 2016; Larsen et al., 2019; Jiménez-Bambague et al., 2021). IBU was removed similarly (but limited) in 1GA + 1CY and 3GA + 1CY treatments. IBU was completely removed when bacteria were added. Likely, heterotrophic bacteria contributed to IBU removal efficiency under carbon limitation (Fortunato et al., 2016; Hasan et al., 2021).

CBZ was only tested in the flask experiments and found recalcitrant as previously reported (García-Galán et al., 2020a; Wu et al., 2022; Wu et al., 2023). This is because of its highly stable aromatic rings (Wang and Wang, 2017).

CLA was only tested in chemostat experiments and showed similar removal efficiencies (77 to 84%) in batch mode of all three treatments. During the steady state of continuous mode, CLA removal efficiency was 72% in GA treatment, which was slightly lower than the batch mode, and 94% in 4GA + 3CY + 3DT treatment. When bacteria were added, the removal efficiency was 83%, 10% lower than 4GA + 3CY + 3DT treatment. These high removal efficiencies are in line with our previous studies and others (Prosenc et al., 2021; Wu et al., 2023).

Overall, species richness had limited effect on the removal of most OMPs. In the flask experiments, increasing microalgal species led to 0 to 20% increase of the removal efficiencies of all OMPs. Adding BAC resulted in >50% increase for BTZ and IBU, but 5 to 29% for other OMPs. In the batch and continuous mode of chemostat experiments, increasing microalgal species richness increased 20 to 49% removal efficiencies of BTZ, DCF, and MCPA, but minor changes were seen for other OMPs. Adding BAC slightly affected the removal efficiencies of all tested OMPs.

## 5.4 Conclusion

In the flask experiments, CAF and SUL were removed >77% in all treatments, while CBZ was not removed under all the tested conditions. MET and BTZ were not removed in the treatments with only microalgae. When bacteria were added, 29% of MET and 57% of BTZ were removed as they were used by bacteria as carbon sources under carbon limitation. The photodegradable compounds (DCF and IBU) were removed similarly in the treatments of 1GA + 1CY (37%) and 3GA + 1CY (54%). MCPA and HYD were removed <40% in the treatments with only microalgae. Furthermore, a positive effect of microalgal species richness was seen in MCPA and HYD. When adding bacteria, the increase was 5 to 12% for the removal efficiencies of DCF, MCPA and HYD, while 50% for IBU.

In the chemostat experiments, removal efficiencies of all six OMPs were similar or slightly higher during the steady state of continuous mode than during batch mode. In batch mode and the steady state of continuous mode, SUL, MET and CLA were removed > 75% in the treatments with only microalgae, with no effect of microalgal species richness. BTZ, DCF, and MCPA ranged from 8 to 40% in 4GA treatment. An increase of 20 to 49% in 4GA + 3CY + 3DT treatment was seen for their removal efficiencies. Additionally, adding bacteria showed a decrease (0 to 15%) in their removal efficiencies.

In conclusion, the confirmation that microalgal species richness with and without bacteria addition increases OMPs removal, as indicated in literature, was not confirmed in this study. The interferences during the experiments (carbon and nutrient limitation and undesired dilutions), together with the lack of replication in the chemostat experiments, resulted in a less clear effect. We could however conclude that species diversity, taxa diversity, and species interactions might play a role in OMPs removal and should therefore be further researched.



# Acknowledgements

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# SUPPLEMENTARY MATERIAL TO CHAPTER 5

**Table S5.1** Compositions of media applied in the flask and chemostat experiments.

| **Compositions** | **Concentration (mg/l)** | |
|---|---|---|
| **Macronutrients** | **Flask** | **Chemostat** |
| $(NH_4)_2SO_4$ | 470 | 340 |
| $K_2HPO_4$ | 56 | 48 |
| $MgSO_4.7H_2O$ | 75 | 75 |
| $CaCl_2.\ 2H_2O$ | 36 | 36 |
| EDTA ferric sodium salt | 8.4 | 8.4 |
| $Na_2EDTA.\ 2H_2O$ | 1.8 | 1.8 |
| $Na_2SiO_3.3H_2O$ | - | 30 |
| **Micronutrients** | **Concentration (mg/l)** | |
| $H_3BO_3$ | 2.86 | 2.86 |
| $MnCl_2.\ 4H_2O$ | 8.1 | 8.1 |
| $ZnSO_4.\ 7H_2O$ | 0.44 | 0.44 |
| $CuSO_4.\ 5H_2O$ | 0.079 | 0.079 |
| $Na_2MoO_4.\ 2H_2O$ | 0.22 | 0.22 |
| $Co(NO_3)_2\ .6H_2O$ | 0.05 | 0.05 |
| **Vitamin** | **Concentration (mg/l)** | |
| Thiamine · HCl | 1.00E-04 | 1.00E-04 |
| Biotin (vitamin H) | 1.00E-06 | 1.00E-06 |
| Vitamin B12 | 1.00E-06 | 1.00E-06 |
| **Carbon source** | **Concentration (g/l)** | |
| Sodium Acetate - Trihydrate | 4.21 | - |
| Meat extract | - | 0.52 |
| Peptone | - | 0.74 |
| **Buffer** | **Concentration (g/l)** | |
| HEPES | 23.83 | - |

**Table S5.2** SPE recoveries (%) of adsorbed OMPs to the biomass in the flask and chemostat experiments.

| OMPs | Internal standards | Flask | Chemostat |
|---|---|---|---|
| CAF | CAF-13C3 | 85 ± 4 | - |
| SUL | SUL-D4 | 84 ± 5 | 83 ± 3 |
| MET | MET-D7 | 58 ± 1 | 59 ± 9 |
| BTZ | BTZ-D4 | 75 ± 2 | 53 ± 4 |
| DCF | DCF-D4 | 102 ± 8 | 50 ± 2 |
| MCPA | MCPA-D3 | 62 ± 1 | 82 ± 1 |
| HYD | HYD-D2 | 84 ± 3 | - |
| IBU | IBU-13C3 | 66 ± 4 | - |
| CBZ | CBZ-13C6 | 57 ± 0 | - |
| CLA | CLA-D3 | - | 88 ± 2 |

**Table S5.3** Quechers recoveries (%) of OMPs in the flask and chemostat experiments.

| OMPs | Internal standards | Flask | Chemostat |
|---|---|---|---|
| CAF | CAF-13C3 | 57 ± 1 | - |
| SUL | SUL-D4 | 70 ± 2 | 63 ± 2 |
| MET | MET-D7 | 72 ± 3 | 74 ± 3 |
| BTZ | BTZ-D4 | 75 ± 2 | 60 ± 3 |
| DCF | DCF-D4 | 78 ± 0 | 50 ± 2 |
| MCPA | MCPA-D3 | 102 ± 8 | 82 ± 1 |
| HYD | HYD-D2 | 53 ± 3 | - |
| IBU | IBU-13C3 | 80 ± 0 | - |
| CBZ | CBZ-13C6 | 56 ± 2 | - |
| CLA | CLA-D3 | - | 49 ± 2 |

**Table S5.4** Bioadsorption (%) of 10 OMPs in the flask and chemostat experiments.

| OMPs | Flask | Chemostat |
|---|---|---|
| CAF | 0.4 ± 0.0 | - |
| SUL | 0.0 ± 0.0 | 0.1 ± 0.0 |
| MET | 0.6 ± 0.1 | 0.2 ± 0.1 |
| BTZ | 0.8 ± 0.2 | 0.1 ± 0.0 |
| DCF | 0.8 ± 0.1 | 0.2 ± 0.2 |
| MCPA | 0.8 ± 0.3 | 0.3 ± 0.3 |
| HYD | 2.7 ± 0.4 | - |
| IBU | 0.5 ± 0.1 | - |
| CBZ | 2.2 ± 0.3 | - |
| CLA | - | 0.2 ± 0.1 |

**Table S5.5** Bioaccumulation (%) of 10 OMPs in the flask and chemostat experiments.

| OMPs | Flask | Chemostat |
|---|---|---|
| CAF | 0.3 ± 0.1 | - |
| SUL | 0.1 ± 0.0 | 0.0 ± 0.0 |
| MET | 0.6 ± 0.4 | 0.0 ± 0.0 |
| BTZ | 0.9 ± 0.2 | 1.2 ± 0.3 |
| DCF | 0.1 ± 0.0 | 0.2 ± 0.0 |
| MCPA | 0.1 ± 0.1 | 0.1 ± 0.0 |
| HYD | 0.0 ± 0.0 | - |
| IBU | 0.1 ± 0.0 | - |
| CBZ | 0.4 ± 0.1 | - |
| CLA | - | 0.8 ± 0.1 |

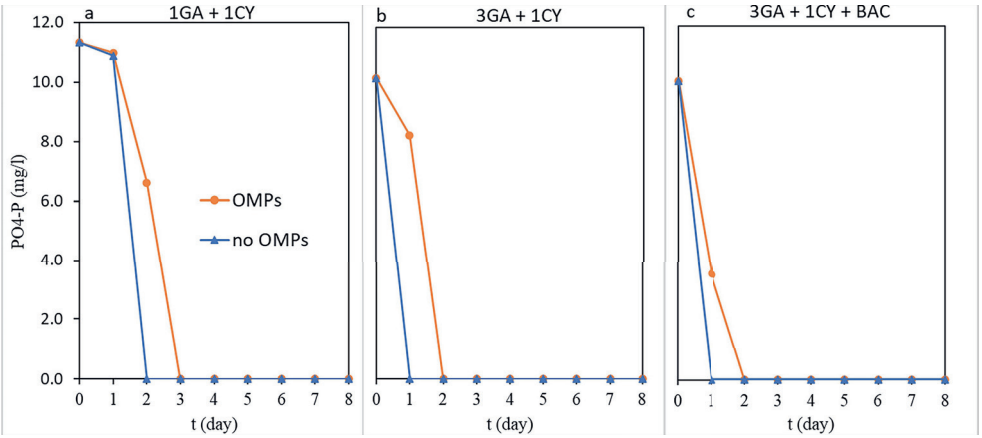


**Figure S5.1** $PO_4^{3-}$-P concentration in time in the chemostat experiments.

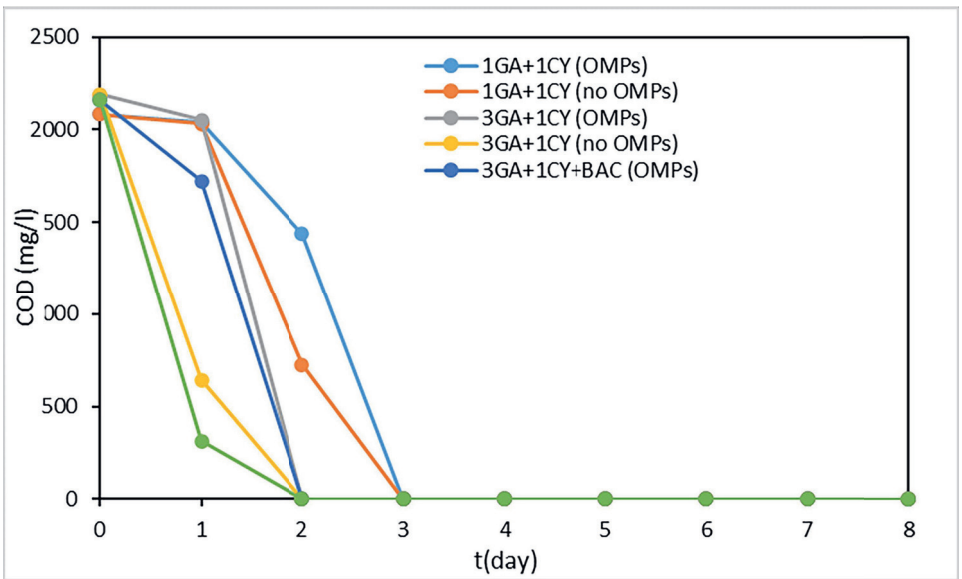


**Figure S5.2** COD concentration in time in the flask experiments.

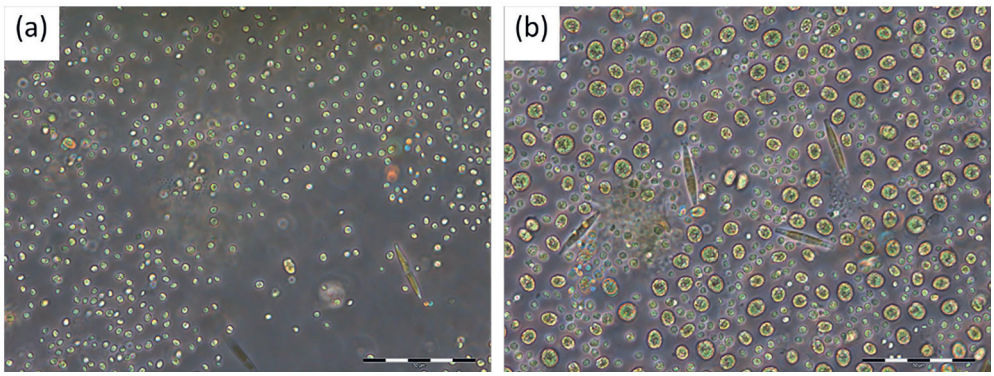


**Figure S5.3** Microscopic images (magnification: 400x) of microalgae in the treatments of 4GA + 3CY + 3DT (a) and 4GA + 3CY + 3DT + BAC (b) during the steady state of continuous mode.

# Chapter 6

# General discussion

# 6.1 Introduction

Current wastewater treatment plants need to be developed towards sustainable facilities with the efficient removal of pollutants, e.g. OMPs and bulk COD, and the effective recovery of available elements, e.g. nitrogen and phosphorus (Angelakis and Snyder, 2015; Soares, 2020). Microalgae-based technologies can efficiently remove a wide range of OMPs from wastewater and retain nutrients into the biomass for further application as fertilizers, bioplastics and other value-added products (Padmaperuma et al., 2018; Liu et al., 2021; Goh et al., 2022).

In this thesis, the removal of OMPs was studied in microalgae-based bioreactors treating various types of aqueous media. The first study focused on the removal processes (biodegradation, bioadsorption, bioaccumulation, and photodegradation) of 6 persistent OMPs during cultivation of a single microalgae species (*Scenedesmus obliquus*). This was performed in artificial medium in flasks in batch mode under artificial light (**Chapter 2**). The main finding of this research was that biodegradation and photodegradation were the predominant removal processes of the tested OMPs. In follow up experiments, the removal efficiency of 16 OMPs was studied during the cultivation of a single, but different, microalgae species (*Chlorella sorokiniana*). This was carried out in a photobioreactor under artificial light, in batch and subsequently in continuous mode (**Chapter 3**). This time, three types of wastewater (anaerobically digested black water, municipal wastewater and secondary clarified effluent) were tested. This study showed that soluble COD and nutrient concentrations predominantly affected the removal of OMPs. Finally, the effect of species richness on OMPs removal was assessed in flasks (batch mode) and photobioreactors (batch and continuous mode) in wastewater and synthetic media, under natural (**Chapter 4**) and artificial light (**Chapter 5)**. The main findings from these studies were that increasing species richness might increase the removal of OMPs. The knowledge gathered in this thesis has deepened our understanding of the removal processes of OMPs by microalgae-based technologies. It has also shed some light on the effects of wastewater characteristics, natural light conditions, and species richness on the removal efficiency of OMPs.

In this chapter, I discuss these findings in a thematic way, to explore the state of art of this technology for the removal of OMPs from (waste)water. The themes are, i) effect of OMPs on microalgal growth, ii) OMPs removal processes, iii) Effect of wastewater characteristics on OMPs removal, iv) Effect of residence time on OMPs removal, v) Effect of species richness on OMPs removal. Moreover, I deduce from these discussions the further developmental needs for future microalgae-based technologies, in treating wastewater to remove OMPs.

# 6.2 Effect of OMPs on microalgal growth

Microalgal biomass is important for the removal of OMPs. In the system with individual species, more biomass can provide more enzymes for the removal of OMPs (**Chapter 3**). In the system with mixed community, a higher proportion of microbes with high removal capacities of OMPs in the biomass can also lead to higher removal. Investigating microalgal growth therefore is necessary for understanding the mechanisms of OMPs removal.

Exposure to OMPs can lead to higher or lower microalgal growth as shown in Table 6.1 and in many studies (Yang et al., 2008; Akiyoshi et al., 2013; Le et al., 2022). The factors influencing this effect are OMPs type, concentration, the presence of OMPs as individual compound or as a mixture, medium, and operational mode (batch or continuous mode).

**Table 6.1** Overview of OMPs effect on microalgal growth in this thesis **(Chapters 2 and 3).**

| Microalgae | Medium | Operational mode | Growth effect | | | OMPs |
|---|---|---|---|---|---|---|
| | | | Stimulation | No effect | Inhibition | |
| *Scenedesmus obliquus* | BG-11 | Batch | | √ | | BTZ (300 µg/l) |
| | | | | √ | | MET (300 µg/l) |
| | | | | √ | | CBZ (1000 µg/l) |
| | | | | √ | | DCF (1000 µg/l) |
| | | | | √ | | MCPP (1000µg/l) |
| | | | | | √ | CLA (60 µg/l) |
| | | | | √ | | Mixture of all 6 OMPs |
| *Chlorella sorokiniana* | AnBW | Batch | √ (Slight) | | | Mixture of all 16 OMPs*(6 µg/l for each) |
| | MW | | | √ | | |
| | SCE | | | √ | | |
| | AnBW | Continous | | √ | | |
| | MW | | | √ | | |
| | SCE | | | | √ (Slight) | |

*All 16 OMPs include caffeine (CAF), benzotriazole (BTZ), sulfamethoxazole (SUL), furosemide (FUR), propranolol (PRO), hydrochlorothiazide (HYD), 4/5-methylbenzotriazole (MeBT), clarithromycin (CLA), trimethoprim (TRI), irbesartan (IRB), metoprolol (MET), carbamazepine (CBZ), diclofenac (DCF), mecoprop (MCPP), 2-methyl-4-chlorophenoxyacetic acid (MCPA).



When OMPs were present individually, it was evident that CLA (present at the lowest concentration of 60 µg/l) showed a higher inhibitory effect on *Scenedesmus obliquus*, than BTZ (300 µg/l), MET (300 µg/l), CBZ (1000 µg/l), DCF (1000 µg/l) and MCPP (1000 µg/l) (**Chapter 2**). Macrolide antibiotics, such as clarithromycin azithromycin and dirithromycin, can inhibit P450 enzymes by the complexation of macrolides and P450 enzymes, and damage the photosystem II (Westphal, 2000; Almeida et al., 2021). These enzymes play an important role in maintaining the substrate- and energy-cycles of microalgae (Hausjell et al., 2018; Zheng et al., 2022). This inhibitory effect can be enhanced when CLA concentration increases. Guo et

al. (2020) demonstrated that clarithromycin at 5 µg/l slightly inhibited the growth of *Chlorella vulgaris and Raphidocells subcapitata,* and that their growth was significantly inhibited when clarithromycin concentration increased to 80 µg/l.

The presence of other OMPs can reduce or increase the inhibitory effect of individual compounds. When CLA was present with BTZ, MET, CBZ, DCF and MCPP, no inhibitory effect on the growth of *Scenedesmus obliquus* was found (**Chapter 2**). Similarly, the mixture of caffeine and ciprofloxacin showed less inhibition on the growth of *Raphidocelis subcapitata* than individual ciprofloxacin (Diniz et al., 2021). On the other hand, when OMPs from the same category were collectively present, the inhibitory effect on microalgal growth can be enhanced. This effect was demonstrated for mixtures of two antibiotics, e.g. trimethoprim and sulfonamides, tylosin and tetracyclines, sulfamethazine and sulfamethoxazole (Yang et al., 2008; Xiong et al., 2019). The authors proposed that the similar toxification behavior of these antibiotics resulted in a joint toxicity effect. Therefore, the mixture effect was affected by the properties of other co-existing compounds.

Wastewater characteristics and operational modes influenced the effect of OMPs on algae growth in this thesis. These two factors affected the removal of OMPs, resulting in varying exposure of OMPs concentrations and growth effects. In batch mode the hormesis effect of a mixture of 16 OMPs on the growth of *Chlorella sorokiniana* was only seen in AnBW with the highest removal of OMPs, compared to SCE and MW (**Chapter 3**). Previous studies have shown that OMPs at low concentrations, such as 0.5 µg/l of sulfamethoxazole and 0.5 µg/l of erythromycin, also had a hormesis effect on the growth of microalgae (Pomati et al., 2004; Yang et al., 2008). In comparison with batch mode, continuous mode allows more sufficient contacts between microalgae and OMPs, possibly resulting in the slight inhibitory effect in SCE in continuous mode (**Chapter 3**). To comprehensively elucidate the impact of wastewater characteristics and operational modes, experiments with more replications (>3) in more types of wastewater are needed.

Overall, the effect of OMPs on microalgal growth can be influenced by the properties of OMPs, the environmental parameters, and the operational parameters. These effects of OMPs can partly indicate the metabolic response of these organisms to

OMPs, which is useful for elucidating the mechanisms of OMPs removal in microalgae-based systems.

# 6.3 OMPs removal processes

This thesis shows that microalgae-based technology has the potential to effectively remove mixtures of OMPs from (waste)water. Twelve out of sixteen OMPs were removed >30% from microalgae-based systems, except for TRI, MCPP, MCPA and CBZ. Remarkably, CAF, SUL, IBU and FUR were completely removed under certain conditions. The removal processes of OMPs in our microalgae-based systems include biodegradation, biosorption, and photodegradation. Additionally, the high deviations in the removal efficiencies of most OMPs indicate the considerable effect of environmental conditions, including wastewater characteristics, operational parameters, light sources, and microorganisms in this thesis.

The mechanisms of different processes that can be affected by experimental conditions are discussed in this section.

## 6.3.1 Biodegradation

Biodegradation was the main removal process of several OMPs (CAF, SUL, IBU, BTZ, CLA, MCPA, and HYD) in this thesis (**Chapter 2 and 5**) and other studies (Gatidou et al., 2019; Xiong et al., 2019; Diniz et al., 2020; Kiki et al., 2020; Usmani et al., 2020; Vassalle et al., 2020; Zhou et al., 2022). By biodegradation, parent OMPs that are susceptible to biomineralization can be converted into $CO_2$ and water; thus improving the chemical and ecological quality of treated water (Nguyen et al., 2020; Usmani et al., 2020). However, biodegradation can also be incomplete, and can result in the formation of toxic transformation products (Kosjek et al., 2009; Wojcieszyńska et al., 2023). Hence, it is important to understand the mechanisms and control the extent of biodegradation of OMPs, for designing microalgae-based OMPs removal processes.

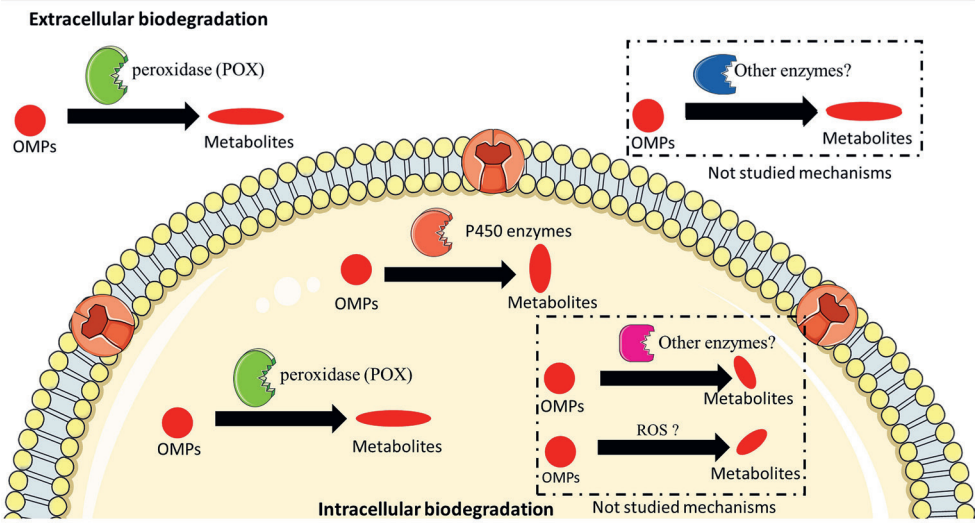


**Figure 6.1** Proposed mechanisms for the initiation of biodegradation of OMPs, adapted from Xiong et al. (2018). Parts of the figure were drawn using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

The conceptual model shows that biodegradation of parent OMPs can be mediated by intracellular and/or extracellular enzymes (Figure 6.1). P450 enzymes and peroxidase (POX) are two types of important enzymes involved in the initiation of the biodegradation by attacking the parent OMPs (**Chapter 2**).

P450 enzymes are responsible for biodegradation of BTZ and CLA (**Chapter 2**). Other biodegradable OMPs (CAF, SUL, IBU, and HYD) tested in the flask experiments of **Chapter 5** can also be degraded by these enzymes (Flockhart and Tanus-Santos, 2002; Asimakopoulos et al., 2013; Chen et al., 2020; Chu et al., 2022; Jia et al., 2020; Mokkawes and De Visser, 2023). Due to the similarities of the biodegradation mechanisms, these OMPs could compete for the binding sites of P450 enzymes (Shou et al., 1994, 2001), resulting in a lower removal efficiency for some of them. This competition effect might have occurred between BTZ and CLA and resulted in a decrease of BTZ removal efficiency when they were present

together (**Chapter 2**). CLA biodegradation was not affected, indicating that CLA had a higher affinity to these enzymes (**Chapter 2**). The varying affinities of different OMPs to P450 enzymes can result in various biodegradation kinetics in a mixture of OMPs. Therefore, the information of OMPs affinities is valuable for understanding the biodegradation mechanisms of a mixture of OMPs.

POX can also initiate the biodegradation of BTZ (**Chapter 2**) and other OMPs, such as CAF, SUL, IBU, MCPA, and HYD (Rubio and Sperelakis, 1972; Wu et al., 1996; Chauhan and Sahoo, 1999; Almaqdi et al., 2019; Vo et al., 2020; Zhang and Geißen, 2010). Additionally, POX is an indicator of oxidative stress of microalgal cells (Aderemi et al., 2018; Vo et al., 2020). POX are therefore produced at high oxidative stress and mediated the biodegradation of OMPs. This thesis shows that POX concentrations, intracellularly as well as extracellularly, were higher in the presence of BTZ as an individual compound, than when BTZ was part of an OMPs mixture (**Chapter 2**). This indicates that POX was responsible for BTZ biodegradation, when BTZ was present as individual compound (**Chapter 2**).

Further, other enzymes and mechanisms can also initiate the biodegradation of OMPs. Laccases can be produced by microalgae, such as *Chlamydomonas moewusii* and *T. aeria*, and initiate biodegradation (Brasil et al., 2017; Usmani et al., 2020). Laccases belong to versatile ligninolytic enzymes (Brasil et al., 2017; Usmani et al., 2020). They can oxidase a wide range of aromatic and nonaromatic OMPs, such as triclosan, bisphenol A, and nonylphenol, with oxygen as co-substrate and water as the only byproduct (Catherine et al., 2016; Usmani et al., 2020). Additionally, reactive oxygen species (ROS), such as the hydroxyl radical, that are generated along with POX, are rather a-specific and can remove a wide range of OMPs (Mirzaei et al., 2017). The ROS-mediated oxidative reactions with OMPs may be inhibited by intracellular substances, such as lipids and protein, as they can scavenge the ROS (Ugya et al., 2020). To further explore this biodegradation mechanism, a ROS-responsive fluorescent probe can be a useful tool to be included in microalgal cultures (Lü, 2017).

A combination of these mechanisms is likely to induce an efficient biodegradation of most OMPs in this thesis. However, biodegradation of these OMPs was not complete and might generate toxic transformation products. For example, hydroxy-

diclofenac was generated during DCF biodegradation by green algae (Liakh et al., 2023), and was toxic to aquatic organisms, like fish, due to the remaining aromatic rings (Wojcieszyńska et al., 2023). Therefore, it is important to optimize the biodegradation processes in microalgae-based systems to achieve a complete mineralization of OMPs.

## 6.3.2 Bioadsorption and bioaccumulation

Bioadsorption and bioaccumulation can be an important intermediate step in the intracellular biodegradation of OMPs, as described in the general introduction. Bioadsorption and bioaccumulation accounted in total for less than 5% of removal efficiency of OMPs (**Chapter 2, 4 and 5).** Higher values were only found in batch experiments with *Scenedesmus obliquus* (1.5% adsorption, 10% bioaccumulation) (**Chapter 2**). The poor bioadsorption and bioaccumulation of OMPs to microalgal biomass has also been documented in previous studies (Gojkovic et al., 2019; Escudero, 2020; Zhang et al., 2021). The low amount of OMPs adsorbing and remaining in the biomass has a positive implication, since the further use of microalgal biomass as fertilizer may not be hampered by OMPs bioaccumulation to algal biomass.

## 6.3.3 Photodegradation

Different types of light (LED light, fluorescence light, and natural light in Netherlands) were applied to cultivate microalgae in this thesis, to identify photodegradation of OMPs, as discussed below.

Direct photodegradation of DCF and IBU was found under fluorescent light (**Chapter 2 and 5**). OMPs with electron-rich functional groups, such as DCF with chlorinated aromatic ring, are more likely to undergo direct photodegradation (Boreen et al., 2003; Muszyński et al., 2020). Furthermore, photodegradation requires a specific wavelength of light for OMPs, which is indicated by the fact that DCF photodegradation only occurred under fluorescent light, and not LED light (wavelength: 400 to 800 nm) (**Chapter 2 and 3**). Additionally, previous studies showed that all the target OMPs in this thesis are photodegradable under UV light with specific wavelength, or under natural light (Calisto et al., 2011; Lei et al., 2022;

Uzelac et al., 2022; Wu et al., 2022). The OMPs recalcitrant to photodegradation under fluorescence light (CAF, SUL, MET, MCPP, MCPA, CBZ, HYD and BTZ) were not subjected to the appropriate wavelength in our study (**Chapter 2 and 3**).Specially, when natural light was applied with a wide range of spectrum (300 to 900 nm), OMPs, such as BTZ and MCPA, cannot be photodegradable. Their required wavelengths (250 to 300 nm for BTZ, 250 to 310 nm for MCPA) are slightly or not included (Zertal et al., 2001; Burrows et al., 2002; Bianco et al., 2016; Wagner et al., 2020). Additionally, photodegradation requires sufficient photons. DCF was >80% removed by photodegradation at natural light conditions in previous studies (Poiger et al., 2001; Koumaki et al., 2015). However, insufficient available photons in our photobioreactors resulted in the absence of DCF photodegradation under natural light conditions (**Chapter 4**). The absence of DCF photodegradation is indicated by the fluctuations and even negative DCF removal efficiency in the first 16 days with similar photo flux density (**Chapter 4**). Thus, supplementing extra light sources can be a feasible strategy to remove OMPs that are recalcitrant to biodegradation, but sensitive to photodegradation under natural light conditions.

Indirect photodegradation, mediated by Iron III, might have contributed to the removal of OMPs, such as CLA under fluorescent light (**Chapter 2**). $Fe^{3+}$ present in BG-11 medium might form a photochemically active complex of $Fe^{3+}$-CLA, resulting in an indirect CLA photodegradation. Other transition metals in our medium, including $Cu^{2+}$ and $Zn^{2+}$, can also interact with CLA, leading to indirect photodegradation (Hamdan, 2003; Vione et al., 2009). However, only the interaction between Fe and CLA was only taken in account, due to the high concentration of Fe, compared to the other metals.

Overall, the photodegradation of OMPs was affected by OMPs type, the wavelengths and intensity of light, and $Fe^{3+}$ in this thesis.

# 6.4 Effect of wastewater characteristics on OMPs removal

The wastewater characteristics that were studied in this thesis and influence OMP removal are soluble COD concentration, and nutrient ($NH_4^+$-N and $PO_4^{3-}$-P) concentration.

## 6.4.1 Effect of soluble COD

This thesis shows the predominate effect of soluble COD on the overall removal of 16 OMPs when growing *Chlorella sorokiniana* in continuous mode (**Chapter 3**). It was proposed that soluble COD in the tested wastewater contained humic substances (Zhou et al., 2020; Zhu et al., 2023), and their quinone moieties positively affected the OMPs removal by acting as electron shuttles to stimulate the biodegradation (**Chapter 3**). OMPs removal in this experiment was steered towards biodegradation and biosorption, excluding photodegradation, since LED light (400 to 800 nm) was used. When light sources with UV wavelengths are used for cultivating algae, soluble COD might interfere with the photodegradation of OMPS, thereby affecting the total removal of OMPs. Soluble COD might enhance the photodegradation of OMPs as photosensitizers (He et al., 2018; Huang et al., 2022). On the other hand, the photodegradation of OMPs can decrease due to the competition for the available photons between photodegradable OMPs and soluble COD (He et al., 2016; Ye et al., 2019). It is interesting to investigate the role of soluble COD on OMPs removal in microalgae-based systems with different light sources, like fluorescent light and natural light. This information will be useful for designing and optimization of microalgae-based processes for the removal of OMPs that are recalcitrant to biodegradation, but sensitive to photodegradation from wastewater.

## 6.4.2 Effect of nutrient

A higher nutrient availability of wastewater was found to increase the overall removal efficiencies of 16 OMPs, which was paralleled by generating more biomass, in the continuous systems with *Chlorella sorokiniana* (**Chapter 3**). Higher biomass

can absorb more OMPs to the microalgal cells for subsequent biodegradation, by generating more enzymes responsible for biodegradation in the system with single microalgal species (Chan et al., 2006). Interestingly, poor nutrient conditions led to a remarkable removal of CAF, IBU, PRO and CLA under continuous conditions (**Chapter 3**). This was attributed to the assimilation of microalgae to the OMPs (**Chapter 3**). For nitro-aromatic OMPs, such as IBU and PRO, they can serve as extra nitrogen source, as shown in this thesis (**Chapter 3**). For removing these compounds, microalgae-based technologies do not require high nutrient concentrations.

# 6.5 Effect of residence time on OMPs removal

Residence time of OMPs in the system is an important parameter for the design of OMPs removal processes. In batch mode, this is the duration time in batch systems. In continuous mode, this equals the hydraulic retention time (HRT), as most OMPs are present in the liquid phase (see 6.3.2).

## 6.5.1 Effect of batch duration

This thesis shows that a longer batch duration results in higher removal efficiency of OMPs (**Chapter 2 and 5**), possibly because the long batch duration allows sufficient contact time between OMPs and biomass. In an 8 days batch experiment with *Scenedesmus obliquus*, BTZ and CLA biodegradation started from day 4, indicating that an adaptation period is needed for biodegradation of OMPs (**Chapter 2**). Additionally, MET removal efficiency was 75% in the treatments of 18 days, significantly higher than the treatments of 8 days in batch mode (**Chapter 5**). It demonstrates a possible positive effect of long batch duration. On the other hand, improperly long batch duration time might result in nutrient depletion, thereby the decaying of the growth and metabolisms of microalgae. Therefore, to achieve an efficient OMPs removal and maintain a healthy microalgal culture, it is beneficial to operate the reactors without the depletion of nutrients in batch mode.

## 6.5.2 Effect of hydraulic retention time

In this thesis, three different HRTs (0.8, 2, and 5 days) were applied in the continuous systems (**Chapter 3 to 5**), and the data show that the removal efficiencies of OMPs, like BTZ and SUL, were affected by HRT (Figure 6.2). The other tested OMPs (MET, CLA, DCF, and MCPA) did not show a clear effect of HRT on their removal efficiency.

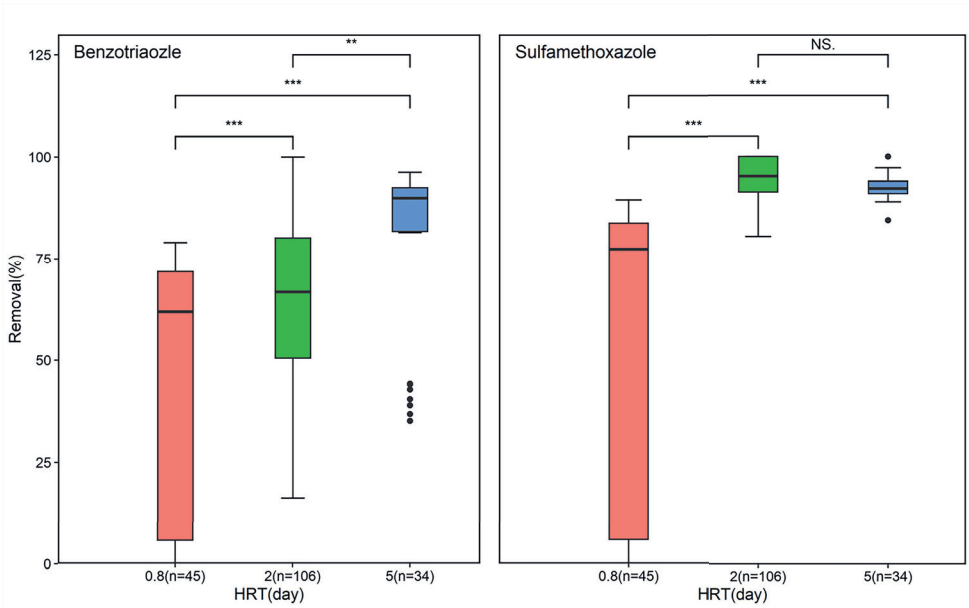


**Figure 6.2** Removal efficiencies of BTZ, and SUL in microalgae-based continuous reactors with three HRTs (0.8, 2, 5 days) in this thesis. The box plot shows the values in maximum, third quartile, median, first quartile, and minimum of non-outlying values. Student's t-test was used to analyze the significance of each paired group for OMP removal, "NS." no significant difference, ** p 0.001 to 0.01, *** p 0.0001 to 0.001, student's t-test.

A longer HRT resulted in higher removal efficiency of both OMPs. The median removal of BTZ increased from 62 to 90%, as HRT increased from 0.8 to 5 days (Figure 6.2). Longer HRT extended the contact time between BTZ and microalgal biomass, resulting in a higher BTZ biodegradation. Matamoros et al. (2016) found

that increasing HRT from 2 to 8 days enhanced removal efficiencies of pesticides (malathion, pentachlorobenzene, chlorpyrifos, and endosulfan) with 20%. The authors linked the improved removal efficiencies in lab-scale reactors with a microalgae consortium grown in agricultural runoff to the increasing contact time between microorganisms and pesticides. In our experiments (Figure 6.2) the median removal of SUL increased with 18% with HRT increasing from 0.8 to 2 days, but a longer HRT of 5 days did not lead to a higher median removal. An HRT shorter than 5 days is apparently sufficient for maximum SUL removal and is consistent with the fact that SUL was easier removed than BTZ in all the continuous systems studied in this thesis (**Chapter 3 to 5**).

To conclude, HRT was an important parameter for OMPs removal efficiency in microalgae-based processes. To achieve maximum removal efficiency of all OMPs, the HRT needs to be fine-tuned to the degradability of the individual OMP, and therefore HRT optimization is recommended for each OMPs mixture present in a specific wastewater. Experiments under controlled conditions need to be conducted for each OMPs mixture to further investigate the effect of HRT on OMPs removal.

# 6.6 Effect of species richness on OMPs removal

To optimize the OMPs removal in microalgae-based systems, the effect of species richness (microalgae and bacteria) was explored (**Chapter 4 and 5**).



This thesis indicates the potential of increasing species richness for OMPs removal optimization (**Chapter 5**). Under natural light conditions, the mixed community with 6 algal species showed higher removal capacities per biomass of most OMPs than *Chlorella sorokiniana* (**Chapter 4**). It appeared that increasing microalgal species richness led to an increase in removal efficiencies of OMPs (**Chapter 5**). Although adding bacteria slightly reduced or did not affect the removal efficiencies of most OMPs in the chemostat experiments, 29 to 53% of increase by adding bacteria was seen for the removal efficiencies of MET, BTZ, and IBU under carbon and phosphorous limitation (**Chapter 5**).

Overall, applying a microalgal-bacterial community with high algal species richness might improve the removal efficiency of OMPs.

# 6.7 Future perspectives on OMPs removal by microalgae-based technologies

## 6.7.1 Effect of species diversity

Species diversity refers to the richness of different species present in an ecosystem and relative abundance of each of these species (Maurer and McGill, 2011). Species diversity is an important factor for many functions of microalgae-based systems, such as biomass growth and lipid production (Weis et al., 2007; Stockenreiter et al., 2012), and possibly OMPs removal. In this thesis, only part of species diversity effect (species richness effect) was assessed. Furthermore, taxonomic richness effect was not included in **Chapter 4** and the flask experiments of **Chapter 5**. In the chemostat experiments of **Chapter 5**, species richness increased with increasing taxonomic richness. The role of taxonomic richness was not clear. Therefore, the aspects related to the effect of taxonomic richness and relative abundance of individual species or taxonomic are discussed below and suggestions are given for future work.

The first suggestion is to investigate the effect of the taxonomic richness on OMPs removal. This effect might have occurred for BTZ removal in chemostat experiment (**Chapter 5**). When cyanobacteria (species richness = 3) and diatoms (3) were added to the treatments with only green algae (4), BTZ removal efficiency increased 29% in batch mode and 49% during continuous mode. Stravs et al. (2019) reported that increasing taxonomic richness showed a significantly higher effect than algal species richness of the same taxa on BTZ removal. The authors found that species from different taxa produce more diverse enzymes for BTZ biodegradation than species from one taxon. In our chemostat experiment, the effect of species richness from one taxon and the effect of taxonomic richness was apparently not apart and needs further investigation. Additionally, the effect of taxonomic richness was not studied in most studies of species diversity effect on OMPs removal (Xiong et al., 2017; Prosenc et

al., 2021; Xiao et al., 2021). Investigating taxonomic richness effect can complete the picture for species richness effect on OMPs removal.

Secondly, the abundance of each species or taxon in the microalgal communities is important to be tracked during the experiments. The overall removal of OMPs can be dominated by the removal capacity of dominated species or taxon in a mixed community. For instance, the dominance of filamentous green algae appeared to decrease the removal efficiencies of IBU, CLA, PRO, BTZ, MeBT, MET, HYD, DCF and IRB in the mixed community (**Chapter 4**). An increase of abundance of certain species or taxon might also affect the removal of OMPs, especially when the species or taxon has superior or poor removal capacities of OMPs in the mixed community. In our batch experiments, the presence of diatoms in a mixed microalgal community with green algae and cyanobacteria negatively affected the degradation of cyprodinil, kresoxim methyl, fipronil, and fludioxonil, possibly due to diatom's lower removal capacities of these OMPs (Stravs et al., 2019). In our chemostat experiments, the treatment with all algal species and bacteria had a higher abundance of diatoms than the treatment with all algal species, resulting in a lower removal of MET (**Chapter 5**). To confirm this possibility, another crucial step is to measure the OMPs removal capacities of each individual species and taxon. This is also useful for identifying if the species richness effect or effect of the abundance of each species dominate the species diversity effect in the mixed community.

Finally, the measurement of enzymes and metabolites is key for unraveling the mechanisms of species diversity effect, as biodegradation of OMPs is enzyme-mediated process (Sutherland and Ralph, 2019; Xiong et al., 2021; **Chapter 2**). So far, most studies of metabolites and enzymes have focused on parts of intracellular biodegradation in microalgae-based systems, including oxidation, reduction, hydrolysis and conjugation reactions (Jia et al., 2020; Chu et al., 2022; Liakh et al., 2023). It is also necessary to investigate the POX-related transformation process, which was also responsible for biodegradation of OMPs in **Chapter 2** and other studies (Vo et al., 2020; Chu et al., 2023).

## 6.7.2 Effect of soluble COD in wastewater

In this thesis, the effect of soluble COD on the overall removal of 16 OMPs was investigated in the system with *Chlorella sorokiniana* (**Chapter 3**). However, the biodegradability and functional groups of soluble COD were not characterized. Soluble COD with various biodegradability and functional groups can have different effects on OMPs removal. Easily biodegradable COD, like acetate and glucose, can stimulate the co-metabolism of OMPs (Vo et al., 2020; Xiong et al., 2020). Recalcitrant COD, such as humic acid, has different mechanisms for influencing the biodegradation of OMPs as described in 6.4.1. Additionally, COD with photoactive functional groups, such as aromatic groups, can trigger indirect photodegradation of photosensitive compounds, such as DCF (He et al., 2016; Liu et al., 2021; Huang et al., 2022). Hence, characterizing the biodegradability and functional groups of COD plays an important role in elucidating the mechanisms of COD effect during the experiments. Furthermore, when using a mixed community of microalgae and bacteria, the effect of COD in wastewater can be more complex due to the varying response of OMPs biodegradation by various species. It is interesting to investigate such a complex effect of soluble COD.

## 6.7.3 Effect of solid retention time

The biological system in WWTP is generally operated under continuous or semi-continuous modes (Angelakis and Snyder, 2015; Abily et al., 2023). HRT and solid retention time (SRT) are both important operational parameters for designing an efficient microalgae-based wastewater system. A long HRT is needed to allow sufficient contact between biomass and OMPs for optimal removal of OMPs (see 6.5.2).

SRT can also influence OMPs removal by affecting the biomass available for the removal of OMPs. This thesis shows that increasing biomass concentration can lead to higher removal of OMPs, especially in the system with single species (**Chapter 3**). Therefore, a long SRT is required to allow sufficient biomass in the bioreactor with single algae species. For the system with mixed community, long SRT can remain the slow growers in the photobioreactor, and maintain a highly diverse community (Kreuzinger et al., 2004; Rios-Miguel et al., 2023). Slow growers, such

as certain diatoms, can be important for removing OMPs in microalgal community (Stravs et al., 2019). On the other hand, SRT needs to be fine-tuned according to chronic toxicity of target OMPs, since an improperly long exposure to OMPs might result in inhibitory effect on microalgal growth (**Chapter 3**). Additionally, in the continuous systems of this thesis, HRT and SRT cannot be adjusted separately to allow both optimal values, as biomass and medium are homogeneously mixed. It can be therefore beneficial to apply continuous system with decoupled HRT and SRT, such as semi-batch systems.

### 6.7.4 Combination of microalgae-based technology with advanced oxidation processes

CBZ was poorly removed in all experimental chapters (**Chapter 2 to 5**) and many other studies (De Wilt et al., 2016; Larsen et al., 2019; Escudero, 2020). Advanced oxidation processes (AOPs), such as ozonation, Fenton, and electrochemical advanced oxidation processes, can efficiently remove CBZ by producing strong oxidative species, like ozone molecular and hydroxyl radicals (Wang and Wang, 2016; Völker et al., 2019; Feijoo et al., 2023). For example, under optimal conditions 99% of CBZ can be removed in 77 min (Feijoo et al., 2023). Therefore, it can be beneficial to combine microalgae-based systems with AOPs, for an efficient removal of a variety of OMPs. Pretreatment by AOPs can decolorize the wastewater and convert complex molecules into easily assimilated compounds for microalgae, therefore resulting in a higher growth in wastewater, especially industrial wastewater and piggery wastewater (Kim et al., 2014; Saranya and Shanthakumar, 2020; Almaguer et al., 2021). This higher growth can also stimulate the removal of OMPs in microalgae-base systems (**Chapter 3**). Hence, pretreatment of AOPs can be a viable addition for optimizing the OMPs removal of microalgae-based technology in wastewater. Additionally, soluble COD can consume a portion of oxidants, when using AOPs for removing OMPs (Hofman-Caris et al., 2017; Van Gijn et al., 2021). This consumption increases the energy demand and operational costs for OMPs removal. AOPs pretreatment apparently is economically feasible for wastewater with low COD concentration. When treating wastewater with high COD concentration, like AnBW, it is more feasible to add AOPs as the post-treatment to minimise the consumption of oxidants. Overall, it is beneficial to combine microalgae-based

technology with AOPs for removing a variety of OMPs. The placement of AOPs needs to be considered based on the COD concentration of wastewater.

# 6.8 Conclusion

In this thesis, the removal processes of OMPs, the effects of wastewater characteristics, residence time and species richness on OMPs removal were investigated to explore the potential of microalgae-based technology. Biodegradation and photodegradation were the main removal processes, while the contribution of bioadsorption and bioaccumulation was negligible (< 5%) for most OMPs. Only CLA showed bioaccumulation, but this was always < 10% in **Chapter 2**. The limited bioadsorption and bioaccumulation of OMPs provide a positive signal for further implication of produced biomass. Biodegradation was mediated by enzymes, like P450 enzymes and POX. Photodegradation was affected by a combination of OMPs type, light wavelength and intensity, and iron in the medium. Furthermore, soluble COD and nutrient concentration predominantly affected the removal of OMPs, especially in the system with single species. Species richness seems to have a positive effect on the biodegradation of OMPs. Moreover, a long batch duration and long HRT favored the degradation of OMPs in microalgae-based system. The removal of OMPs was affected by an interplay of OMPs type, light conditions, wastewater characteristics, species richness and residence time. Overall, microalgae-based technology has the potential for efficiently removing a variety of OMPs.

To further understand and better apply microalgae-based technology for OMPs removal, several aspects need to be considered. First, species diversity appeared to be important for the biodegradation of OMPs by microalgae. To elucidate the role of species diversity, it is needed to investigate the taxonomic richness effect, and track the relative abundance and OMPs removal capacities of each species or taxon in the microalgal or microalgal-bacterial communities during the operations. Additionally, measuring enzymes and metabolites can provide direct evidence for mechanisms of species diversity effect. Another aspect to further investigate is the role of wastewater COD on OMPs removal by characterizing the biodegradability and

functional groups of soluble COD. Further investigation should focus on systems with mixed community. Finally, to achieve an optimal removal of OMPs, two strategies ae proposed. One is to allow optimal HRT and optimal SRT in one reactor by decoupling these two parameters for removing degradable OMPs. Another one is to combine microalgae-based technology with advanced oxidation processes (AOPs) for removing poorly removed OMPs. The knowledge accumulated in this thesis can deepen the understanding the OMPs removal by microalgae-based technology and provide guidance for further optimization and upscaling of this technology in wastewater treatment plant.

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# List of abbreviations

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| AnBW | anaerobically digested black water |
| AOPs | advanced oxidation processes |
| BAC | bacteria |
| BTZ | benzotriazole |
| CAF | caffeine |
| CBZ | carbamazepine |
| CLA | clarithromycin |
| $CO_2$ | carbon dioxide |
| COD | chemical oxygen demand |
| CY | cyanobacteria |
| DCF | diclofenac |
| DOM | dissolve organic matter |
| DT | diatom |
| EPS | extracellular polymeric substance |
| FUR | furosemide |
| GA | green algae |
| $H_2O_2$ | hydrogen peroxidase |
| HPLC | high-performance liquid chromatography |
| HRAP | high rate algal pond |
| HRT | hydraulic retention time |
| HYD | hydrochlorothiazide |
| IBU | ibuprofen |
| IRB | irbesartan |
| LC-MSMS | liquid chromatography tandem mass spectrometry |
| LED | light emitting diode |
| MCPA | 2-methyl-4-chlorophenoxyacetic acid |
| MCPP | mecoprop |
| MeBT | 4/5-methylbenzotriazole |
| MET | metoprolol |
| MW | municipal wastewater |
| OMPs | organic micropollutants |
| PBR | photobioreactor |
| PCA | principal component analysis |
| PFD | photo flux density |

| | |
|---|---|
| POX | peroxidase |
| PRO | propranolol |
| RDA | redundancy dimensional analysis |
| ROS | reactive oxidative species |
| SCE | secondary clarified effluent |
| SPE | solid phase extraction |
| SRT | solid retention time |
| SUL | sulfamethoxazole |
| TRI | trimethoprim |
| UASB | upflow anaerobic sludge blanket |
| UV | ultraviolet |
| WWTP | wastewater treatment plants |

# Summary

Organic micropollutants (OMPs), including pharmaceuticals, personal care products, and pesticides, are currently widely discharged into aquatic ecosystems. Although these OMPs are present from µg/l to ng/l, they can negatively affect the metabolism of target and non-target organisms, thereby inhibiting their growth and in this way influence the functioning of the ecosystem. Conventional wastewater treatment plants (WWTP) are the major emission source of OMPs, since conventional WWTP are not designed for the removal of OMPs. It is, therefore, necessary to develop innovative technologies for the efficient removal of OMPs from wastewater.

Microalgae-based technology can be a sustainable strategy for this purpose. This technology can efficiently remove a variety of OMPs from different types of wastewater by four different removal processes, namely biodegradation, bioadsorption, bioaccumulation and photodegradation. So far, most studies focus on the removal efficiency of OMPs by microalgae-based technologies from synthetic media or one type of wastewater, and often short batch mode experiments are used. Moreover, the information on OMPs removal in photobioreactors operated in continuous mode for a longer time is rare, as well as the impact of wastewater characteristics. Additionally, only few studies on OMPs removal address the aspect of OMPs mixture effect, i.e. that OMPs can enhance or inhibit degradation of other OMPs. Finally, there is a lack of studies comparing the effect of OMPs removal by single species versus mixed microbial communities.

This thesis explores the potential of microalgae-based technology for the removal of OMPs. The removal processes of OMPs were investigated, and the effects of wastewater characteristics and microalgal and bacterial species richness on the removal of OMPs were assessed.

In **Chapter 2**, the contributions of removal processes of six OMPs and the role of peroxidase in the biodegradation of OMPs were investigated in batch mode with *Scenedesmus obliquus* under optimal growth conditions and fluorescent light. The target compounds include diclofenac, clarithromycin, benzotriazole, metoprolol, carbamazepine, and mecoprop, and they were spiked as individual compounds or a mixture. Diclofenac was mainly removed by photodegradation, and clarithromycin was mainly removed by photodegradation and biodegradation. Benzotriazole was

mainly removed by biodegradation. Other OMPs were poorly removed. The contributions of bioadsorption and bioaccumulation were negligible for all OMPs. Furthermore, the photodegradation of diclofenac and clarithromycin was inhibited by the presence of OMPs mixture. Also, the biodegradation of benzotriazole was negatively affected by the presence of OMPs mixture, but this did not occur for clarithromycin. Additionally, intracellular and extracellular POX were involved in biodegradation of benzotriazole in the presence of OMPs mixture. These findings deepen the understanding of removal processes of individual OMPs and the mixture of OMPs.

**Chapter 3** focused on the impact of wastewater characteristics on the removal of sixteen OMPs in the continuous systems (HRT: 0.8 days) with *Chlorella sorokiniana* under LED light. The compounds include caffeine, trimethoprim, propranolol, carbamazepine, sulfamethoxazole, benzotriazole, 4/5-methylbenzotriazole, clarithromycin, irbesartan, metoprolol, diclofenac, ibuprofen, furosemide, hydrochlorothiazide, mecoprop, and 2-methyl-4-chlorophenoxyacetic acid. Anaerobically digested black water (AnBW), municipal wastewater (MW) and secondary clarify effluent (SCE) were selected as media. Eleven tested OMPs were removed from AnBW and MW, but only 3 tested OMPs were removed in SCE. The removal efficiency of most OMPs in AnBW and MW decreased when switching from batch to continuous mode, mostly due to the decrease of biomass concentration. Furthermore, statistical analysis shows that soluble COD concentration and biomass concentration are the predominant factors for OMPs removal during the steady state of continuous mode. Carbon uptake rate of the biomass has a higher effect than nitrogen and phosphate. These findings give an insight on wastewater characteristics effect and highlight the importance of biomass concentration for OMPs removal in microalgae-based systems with single species.

The effect of microalgal and bacterial species richness on OMPs removal in microalgae-based system was explored in **Chapter 4 and 5**.

In **Chapter 4**, the removal of sixteen OMPs in two 27.5L tubular photobioreactors was compared for 112 days under Dutch natural light conditions of spring/summer. One was inoculated with a mixed community of five green microalgal species, one cyanobacteria and heterotrophic bacteria, another one was inoculated with *Chlorella*

*sorokiniana*. Twelve out of sixteen OMPs were removed from both photobioreactors. For these OMPs, the mixed community photobioreactor had higher removal capacities per biomass than *Chlorella sorokiniana* photobioreactor before day 80, indicating the positive effect of species richness. Afterwards, the invasion of filamentous green algae reduced the removal capacities in mixed community, and therefore lower than *Chorella sorokiniana* photobioreactor.

In **Chapter 5**, flask experiments and chemostat experiments were conducted under artificial light. The flask experiments with green algae, cyanobacteria, and heterotrophic bacteria were conducted under fluorescent light with nine OMPs. Light was not limited, but carbon and phosphorous limitation occurred, which might have interfered the species richness effect. The positive effect of microalgal species richness was only seen with diclofenac, hydrochlorothiazide, ibuprofen and 2-methyl-4-chlorophenoxyacetic acid, which was demonstrated by a 17 to 20% increase in the removal efficiency. Adding bacteria resulted in 5 to 29% increase in the removal efficiency of metoprolol, 2-methyl-4-chlorophenoxyacetic acid, hydrochlorothiazide and diclofenac, and >50% for benzotriazole and ibuprofen. The chemostat experiments with 4 green algal species, 3 cyanobacteria, 3 diatoms and heterotrophic bacteria were conducted with six OMPs under LED light. In batch mode, the positive effect of microalgal species richness was only seen in benzotriazole, diclofenac, and 2-methyl-4-chlorophenoxyacetic acid, was demonstrated by a 20 to 49% increase. In continuous mode, positive microalgal species richness effect was also seen in clarithromycin. Furthermore, a slightly negative or no effect was seen when bacteria were added. However, the unexpected dilution in the treatment with only green algae and lack of replication interfered the observations of species richness effect in chemostat experiments. Therefore, experiments with sufficient replications under optimal conditions need to be further applied to investigate the effect of species richness. Nonetheless, the findings of **Chapter 4 and 5** shed a light on the species richness effect on the removal of OMPs.

In **Chapter 6,** the outcomes of all the experiment chapters were summarized, and the future perspectives were discussed. Biodegradation and photodegradation were found to be the predominant removal processes of OMPs in microalgae-based systems. Biodegradation of OMPs can be affected by soluble COD, nutrients, and

species richness. Photodegradation of OMPs is affected by light wavelengths, light intensity, and co-existing substrates, such as $Fe^{3+}$. Additionally, a long batch duration and long hydraulic retention time can benefit the removal of OMPs, by allowing sufficient contact between microorganisms and OMPs.

Finally, it is recommended to further investigate the effect of species diversity on OMPs removal. More specifically, the effect of taxonomic richness and the abundance and contributions of each species/taxon to the removal of OMPs need to be investigated in the microalgal-bacterial community. To further elucidate the species diversity effect, measuring metabolites and enzymes involved in the degradation of OMPs can provide direct information about mechanisms and species involved in the degradation of OMPs. To further investigate the effect of soluble COD on the removal of OMPs, it is suggested to characterize COD and investigate the effect of COD in microalgae-based systems with high species diversity. To achieve an optimal removal of OMPs in microalgae-based continuous systems, decoupling HRT from SRT is recommended: a high SRT allows for larger quantities of biomass in the reactor which can strongly enhance the removal of OMPs. Coupling with advanced oxidation processes (AOPs) can be beneficial for removing recalcitrant OMPs. The knowledge represented in this thesis aims to deepen the understanding of microalgae-based technology for OMPs removal and shed a first light on the next steps for optimizations and application of this technology.

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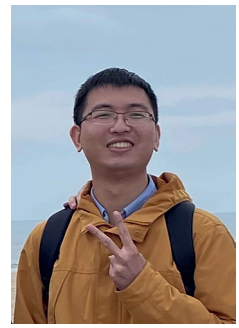
# About the author



Kaiyi Wu was born on 10th September 1993 in Zhanjiang, Guangdong, China. In 2015, he obtained his bachelor's degree in environmental engineering in South China University of Technology (SCUT), Guangzhou, China. During his bachelor, he was involved in two research projects. They are entitled "Nitrogen removal and $N_2O$ emission characteristics during shortcut simultaneous nitrification and denitrification process" and "Removal of As (III) and As (V) from aqueous solutions using nanoscale zero valent iron-reduced graphite". During this period, he developed an interest in wastewater treatment processes.

From 2015 to 2018, he pursued a master's degree in SCUT and worked on industrial wastewater treatment process under the supervision of Pro. Chaohai Wei. His master thesis title is the mineralization of bio-treated coking wastewater by catalytic ozonation.

In 2019, he started his PhD study in department of aquatic ecology of Netherlands institute of ecology (NIOO-KNAW) and sub-department of environmental technology of Wageningen University & Research (WUR), with a scholarship from Guangzhou Elite Project (GEP). He worked on the removal of 16 organic micropollutants (OMPs) from wastewater by microalgae-based technologies. The outcomes of his doctoral study have been described in this thesis.

# Publications

Zhang, F., Wei, C., **Wu, K.**, Zhou, H., Hu, Y. and Preis, S., 2017. Mechanistic evaluation of ferrite AFe2O4 (A= Co, Ni, Cu, and Zn) catalytic performance in oxalic acid ozonation. Applied Catalysis A: General, 547, 60-68.

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- Competence Assessment, Wageningen Graduate Schools (2019)
- Teaching and Supervising thesis students, Wageningen Graduate Schools (2019)
- Scientific Writing, Wageningen Graduate Schools (2020)

**Management and Didactic Skills Training**

- Supervising 4 MSc students with thesis (2019-2023)
- Supervising 1 BSc student with thesis (2022)

**Oral Presentations**

- *Removal processes of individual and a mixture of organic micropollutants in the presence of Scenedesmus obliquus.* 12th IWA Micropol&Ecohazard Conference 6-10 June 2022, Santiago de Compostela, Spain
- *Removal processes of individual and mixed organic micropollutants by microalgae-based technologies.* 13th IWA Specialist Conference on Wastewater Ponds and Algal Technologies, 3-6 July, Melbourne, Australia (Online)

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# Colophon

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