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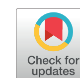
Removal processes of individual and a mixture of organic micropollutants in the presence of *Scenedesmus obliquus*



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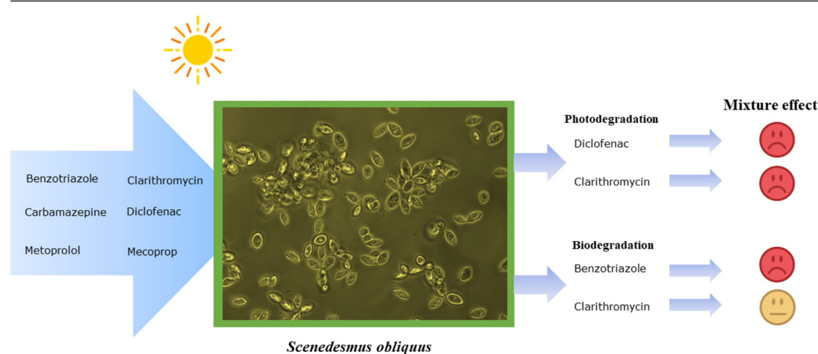
HIGHLIGHTS

- Photodegradation and biodegradation were the main removal processes in our study.
- The used OMP mixture inhibited benzotriazole biodegradation.
- The used OMP mixture did not affect clarithromycin biodegradation.
- Photodegradation of diclofenac and clarithromycin was inhibited by OMP mixture.
- Negligible removal was observed by bioadsorption and bioaccumulation.

GRAPHICAL ABSTRACT



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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. Short experiments (8 days) were performed to avoid masking of OMP 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 these two processes (clarithromycin). Peroxidase was involved in intracellular and extracellular biodegradation when benzotriazole was present as individual compound. Carbamazepine, metoprolol and mecoprop showed no biodegradation or photodegradation, and neglectable removal (<5%) by bioadsorption and bioaccumulation. Using an OMP mixture 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 OMP mixture. This indicates that the presence of OMPs inhibits the photodegradation and biodegradation of some individual OMPs. These results will improve our understanding of removal processes of individual and mixtures of OMPs by microalgae-based technologies for wastewater treatment.

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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 endocrine and other bioregulatory systems of aquatic organisms, such as fish and daphnia (Kristofco et al., 2015; Schwarzenbach, 2006). Currently, OMPs are not sufficiently removed by conventional wastewater treatment plants (WWTP) (Sutherland and Ralph, 2019). Thus, many technologies, such as ozonation or activated carbon, have been developed in the past decade to eliminate OMPs from wastewater (Fundneider et al., 2021; Völker et al., 2019).

Microalgae wastewater treatment is a new technology that combines water cleaning with the 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 OMP removal processes, including photodegradation, biodegradation, bioadsorption and bioaccumulation (Usmani et al., 2020; Xiong et al., 2018). Of these processes, photodegradation can achieve an efficient removal of 40 to 100% of light sensitive compounds, such as ibuprofen and diclofenac (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 (Sutherland and Ralph, 2019; Xiong et al., 2018). 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 biodegradation, 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 (Vo et al., 2020; Xiong et al., 2018). 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 (Hena et al., 2021; Maryjoseph and Ketheesan, 2020). Moreover, the few studies that evaluate these aspects, focus on individual compounds (da Silva Rodrigues et al., 2020; Escapa et al., 2016; 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 responsible for 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 OMP mixture 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. Materials and methods

2.1. Target OMPs

Diclofenac (DCF), clarithromycin (CLA), benzotriazole (BTZ), metoprolol (MPL), 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 wastewaters (Giannakis et al., 2015). The concentrations of OMPs were chosen based on studies with similar experimental conditions, set-up and microalgae 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 MPL 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., 2020; 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 OMP stock solutions in methanol were evaporated by gentle nitrogen gas till dryness, and afterwards the sterilized BG-11 medium was added to get the spiked BG-11 medium.

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 S1) until constant growth rate was reported for a minimum of 7 generations. The adaptation was conducted in 300 ml sterilized Erlenmeyer 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.

2.3. Experimental set-up

The experiments with six individual compounds or the OMP mixture were performed in 300 ml flasks as described at Section 2.2. The experiments lasted only 8 days to prevent light and nutrient limitations. 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 OMP removal processes 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 wrapped with aluminum foil) (Fig. 1). In order to quantify the OMP 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 microalgae 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 experimental run, as no decomposition of the OMPs (D) or microalgae growth in the dark (F) was expected. They were performed to experimentally confirm the expectations.

OMP removal in control C was denoted as photodegradation. Bioadsorption and bioaccumulation were determined as described in Section 2.4. The difference between OMP removal in treatment A and photodegradation, bioadsorption and bioaccumulation was denoted as biodegradation.

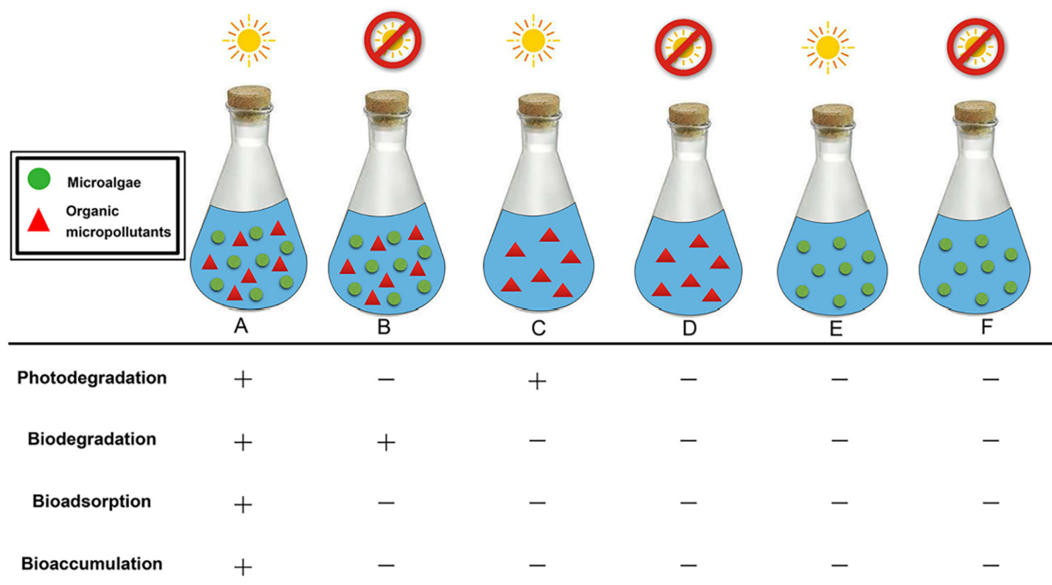

Fig. 1. Schematic representation of the batch experiments with conditions supportive (+) or non-supportive (−) to potential removal processes for each treatment and control.

2.4. OMP extraction

Bioadsorbed and bioaccumulated OMPs were extracted according to the work-flow procedure in Fig. S1. 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 (Xiong et al., 2016). The OMPs released from the pellet to the washing milli-Q water were the bioadsorbed OMPs (Vo et al., 2020; Kiki et al., 2020; Xiong et al., 2016). These OMPs were extracted by solid phase extraction (SPE). The washing milli-Q water and internal standards (Table S2) were loaded on the SPE cartridges (Oasis HLB 6 cc, 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 cartridges were rinsed with 5 ml of milli-Q water to remove any absorbed impurities. 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 ionisation 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). 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, were the bioaccumulated OMPs. These OMPs were extracted by a Quechers standard method (Lehotay, 2007). For this, the pellet and internal standards (Table S2) were transferred to 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 a clean 15 ml 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-MS after centrifugation (4500 rpm, 10 min). The Quechers method showed a recovery of 42 to 111% with a standard deviation up to 15% (Table S2). Bioaccumulation was evaluated by the ratio between the amount of bioaccumulated OMPs and the initial amount of OMPs.

2.5. Analytical methods

The growth of *S. obliquus* in the treatments (A, B) and the controls (E, F) was quantified by measuring chlorophyll *a* and dry weight. Chlorophyll a was measured daily in duplicate using a 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 an 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 a 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, 20–21 min 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 MPL (268.0 → 116.0) were detected in positive ionisation model, and MCPP (213.0 → 141.0) was detected in negative ionisation mode.

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 OMP mixture. 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 30 s, 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, 45,000 rpm, 4 °C). Finally, the supernatant was used for the intracellular enzyme assay.

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.

3. Results and discussion

3.1. Microalgal growth

The chlorophyll *a* showed similar trends in the growth curves for all the OMPs, except for the one with CLA (Fig. 2a). When BTZ, CBZ, MPL, DCF and MCPP were present as individual compounds, similar dry weight at day 8 (1.6 ± 0.1 g/l, Fig. 2b) and specific growth rate (1.0 ± 0.1 d^{-1}, Table S3) were achieved. This shows that BTZ, CBZ, MPL, 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 (Fig. 2), indicating that the growth of *S. obliquus* was inhibited by CLA. In comparison, *S. obliquus* was less tolerant to CLA than *S. quadriculata*, *C. vulgaris* and *R. subcapitata* (Guo et al., 2020; 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., 2020). During this process, CLA can inhibit the activity of enzymes such as P450, which is involved in the biodegradation of OMPs (Akiyoshi et al., 2013; Masubuchi and Horie, 2007). A similar process might occur in *S. obliquus*, since microalgae have similar OMP degradation pathway as bacteria (Méndez García and García de Llasera, 2021).

The dry weight at day 8 and specific growth rate in the presence of the OMP mixture were similar with control E (no OMPs) (Fig. 2, Table S3). This shows that the OMP mixture, including CLA, did not inhibit microalgal growth. Furthermore, the OMP mixture had an antagonistic effect on the CLA-induced inhibition on microalgal growth. Sharma et al. (2021) showed that OMPs compete for the binding sites of cells responsible for growth inhibition. Possibly such a mechanism explains the effect of CLA on microalgal growth.

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) (Fig. 3). In contrast, CBZ, MPL and MCPP showed negligible removal (Fig. S2). Only a small amount (<5%) of these three compounds was bioadsorbed or bioaccumulated by the microalgal biomass (Table S5).

DCF was only removed in the presence of light, with no significant difference in the presence or absence of microalgae (Fig. 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 OMP mixture (Fig. 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 was used (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 (Fig. 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 above the mg/l range is needed to stimulate the DCF biodegradation.

Bioadsorption and bioaccumulation of DCF were negligible (<1.2%). Likewise, another green microalgal species (*Chlorella* 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).

CLA removal showed similar patterns in treatment A (Algae + light) and control C (Light) during the first four days (Fig. 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 (Fig. 3c, d). Photodegradation of CLA accounted for 48% removal

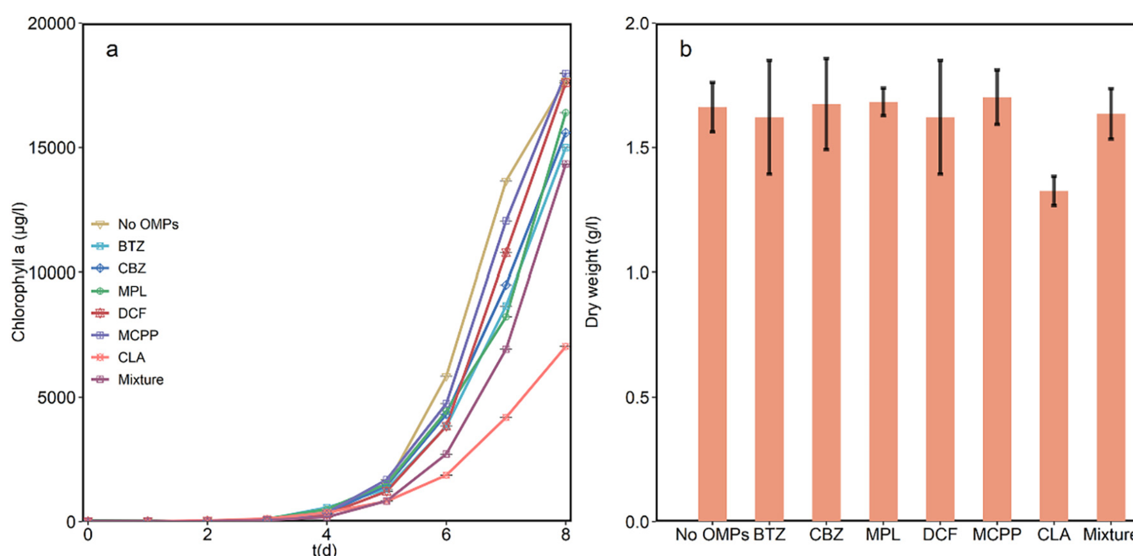

Fig. 2. Dry weight at day 8 (a) and chlorophyll *a* (b) in time in the presence of OMPs (treatment A) and the absence of OMPs (control E) with *S. obliquus*.

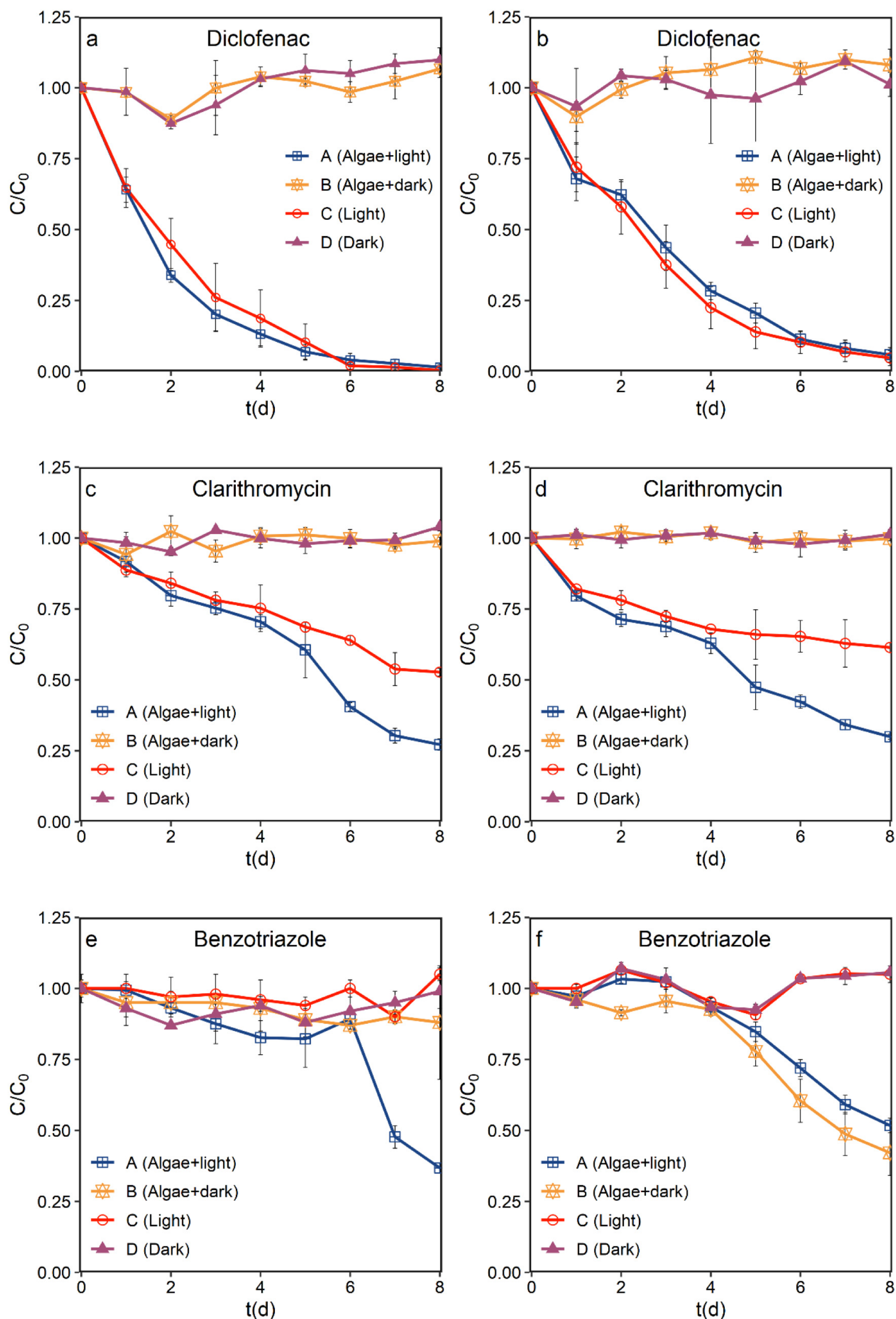

Fig. 3. Relative removal (C/C_0) of DCF (a), CLA (c) and BTZ (e) when present as individual compound; DCF (b), CLA (d) and BTZ (f) when present in the OMP mixture.

when present as individual compound, and 39% removal when present in the OMP mixture (Fig. 4). This shows that CLA was less photodegradable than DCF, which is consistent with another study, focusing on the photodegradation 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

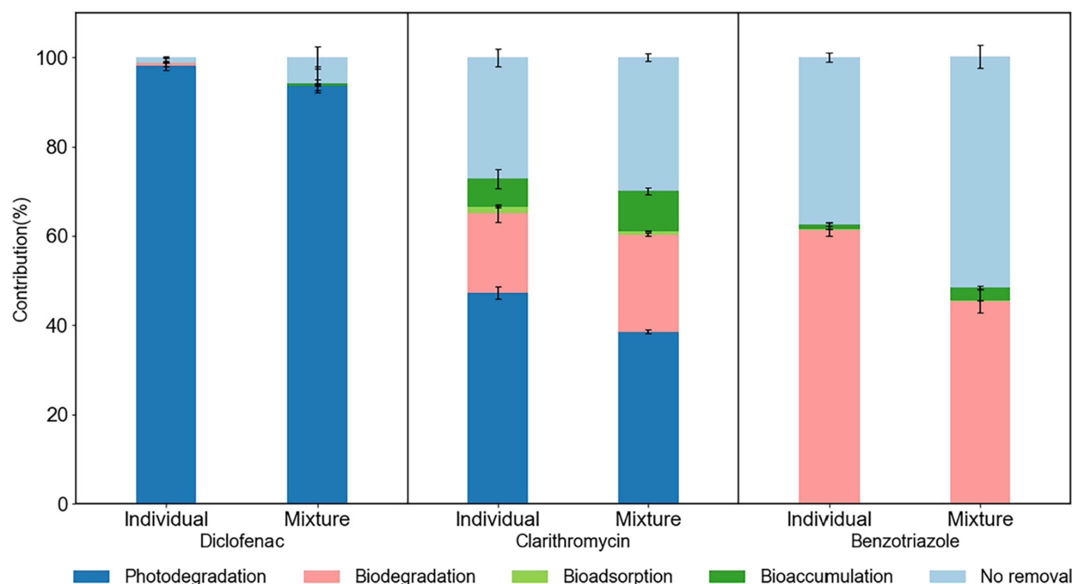

Fig. 4. Contribution of each removal process in treatment A at day 8.

enhance indirect photodegradation via the formation of a Fe^{3+}-clarithromycin complex, which is a photochemically active compound (Kari and Giger, 1995; Mathon et al., 2019; Vione et al., 2009). 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 removal process of CLA in treatment A. Biodegradation accounted for 27% removal of CLA as individual compound or in the OMP mixture (Fig. 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 (Tian et al., 2019; Xiong et al., 2018). Possibly, a similar pathway is involved in CLA biodegradation by microalgae.

Bioadsorption contributed to <1.5% to the removal of CLA, and bioaccumulation accounted for <10% removal of CLA (Fig. 4). The poor bioadsorption percentages of all six OMPs might have been due to the saturation of binding sites on microalgal cells (Sutherland and Ralph, 2019). 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 (Bui and Choi, 2010; Sutherland and Ralph, 2019). This also results in the highest OMP/biomass ratio of CLA when it was present as individual compound (3.5 ± 0.7 μg/g) and in the OMP mixture (3.6 ± 0.3 μg/g).

BTZ removal was only observed in the presence of algae (Fig. 3e, f). In treatment A (Algae + light), bioadsorption and bioaccumulation accounted for <3% removal (Fig. 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 OMP mixture (48%) (Fig. 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, the first-order kinetic constant of BTZ biodegradation in our experiments, when present as individual compound ($k_1 = 0.44\ d^{-1}$) was higher than in the OMP mixture ($k_1 = 0.14\ d^{-1}$) (Table 1). The lower BTZ biodegradation and first-order kinetic constant in the OMP mixture might have been due to the competition with CLA on the binding sites of PY450 enzymes as these enzymes are involved in the biodegradation of BTZ and CLA (Asimakopoulos et al., 2013). However, in the OMP mixture a decrease of CLA biodegradation was not detected. The biomass in the mixed systems was 20% higher than in the individual CLA treatments (Fig. 2b), most likely compensating this competitive inhibition effect.

In treatment B (Algae + dark), BTZ removal was only achieved in the OMP mixture (Fig. 3f). Apparently, a light-independent BTZ biodegradation occurred, which requires the presence of the other OMPs.

MPL removal was not observed in our study, which is in line with previous findings using *Chlorella vulgaris* and a mixed algal population of *Stigeoclonium* sp. and diatoms (Bodin et al., 2016; García-Galán et al., 2020). In contrast, a mixed green algal population dominated by *Tetradesmus dimorphus* showed a complete degradation of MPL in a pilot-scale open bioreactor (Gentili and Fick, 2017). Also bacteria can have played a role in those experiments, since these are known to degrade MPL (He et al., 2018). No bacteria had been added. The microalgae species and the absence of bacteria may have led to the absence of MPL biodegradation in our system.

MCPP was not removed in microalgae-based systems of this study and others (Matamoros and Rodríguez, 2016). In contrast, using an immobilized microalgae-based system achieved 70% biodegradation of

Table 1
Kinetic data of DCF, CLA or BTZ removal ($n = 3$).

OMPs	Conditions	As individual compound		In the OMP 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[b]	0.95[b]	0.14[a]	0.98[a]
	B (algae + dark)	–	–	0.18[a]	0.97[a]
	C (light)	–	–	–	–
	D (dark)	–	–	–	–

[a] Data from day 3 to 8 were used.
[b] Data from day 6 to 8 were used.

MCPP, 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 reported before for microalgae-based systems (de Wilt et al., 2016; Larsen et al., 2019; Matamoros et al., 2016). 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).

3.3. Removal kinetics

The removal kinetics of DCF, CLA, and BTZ were investigated to elucidate the removal rate for various active processes (Table 1), using regression analyses on selected data presented in Fig. 3.

DCF removal in control C (Light) yielded a first-order kinetic constant (k_1) of 0.48 d^{-1} when present as individual compound, which was higher than in the OMP mixture (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 OMP mixture (k_1 = 0.04 d^{-1}). The lower first-order kinetic constant in the experiment with the OMP mixture was possibly because DCF and CLA compete for the available photons during photodegradation. This effect has also been observed in other study 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 in our study (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 in 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 can achieve a comparable biodegradation of BTZ in our system. Additionally, the negligible bioaccumulation and bioaccumulation in microalgae-based system demonstrate that BTZ was removed from the system, instead of being transported to the microalgal biomass (Fig. 4). A microalgae-based system is therefore a competitive alternative to conventional WWTP for BTZ removal.

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).

In treatment A (Algae + light), the highest activity of intracellular and extracellular POX was found in the presence of BTZ as individual compound (Fig. 5). Apparently, the presence of the OMP mixture reduced the production of intracellular and extracellular POX.

POX was reported to metabolise BTZ and its methyl derivatives (Wu et al., 1998). Furthermore, 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 OMP mixture was 75 U/ml/h, four-fold higher than when BTZ was present as individual compound (18 U/ml/h). This is in line with the observed high BTZ removal in treatment B in the OMP mixture (Fig. 3). This shows that POX plays a role in BTZ biodegradation, and that 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 OMP mixture (Fig. 5). The increase of intracellular POX activity was also observed in *R. subcapitata* and *C. vulgaris* after exposure to >20 μg/l of CLA (Guo et al., 2020). However, CLA biodegradation was similar in treatment A with the presence of CLA as individual compound and in the OMP mixture (Fig. 4). POX can not only use OMPs as substrate, but also use ascorbate and glutathione, which are important antioxidants in algae cells (Nicodemus et al., 2020). Possibly, POX did not contribute to CLA biodegradation, but contributed to the oxidation of ascorbate and glutathione.

4. Conclusion

Photodegradation and biodegradation were the prevailing removal processes of DCF, CLA and BTZ in our experiments. For the other three OMPs (MPL, 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 by 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 OMP mixture on photodegradation and biodegradation were identified in our system. The OMP mixture inhibited photodegradation, which is indicated by the lower first-order kinetic constants of photodegradation of DCF and CLA, when present in the OMP mixture. The adverse effect of the OMP mixture on BTZ biodegradation is manifested by the decreased biodegradation and decreased the first-order

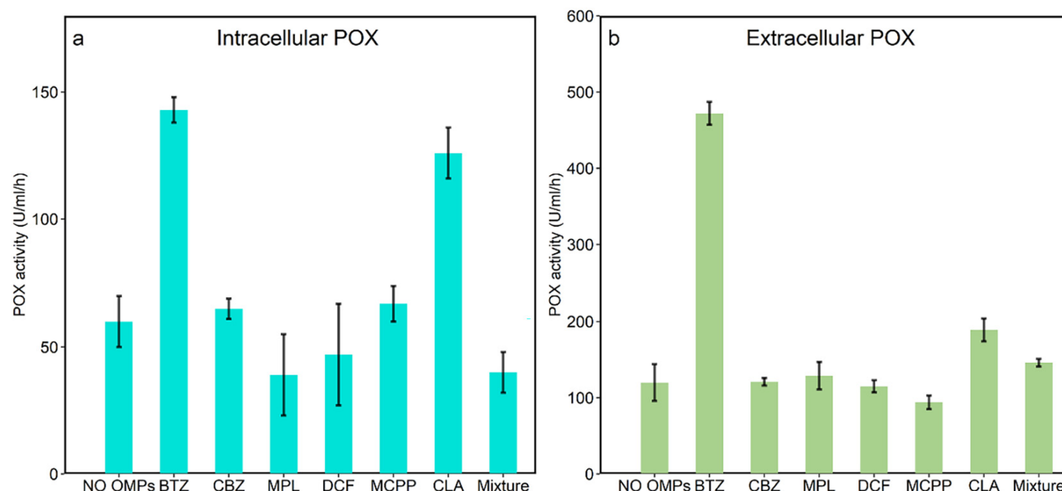

Fig. 5. Activity of intracellular (a) and extracellular (b) POX in the presence of OMPs (treatment A) and absence of OMPs (control E).

kinetic constant in the presence of the OMP mixture. No inhibitory effect of the OMP mixture was observed for CLA biodegradation.

POX was involved in the intracellular and extracellular biodegradation of BTZ when present as individual compound, and did not play a role in the biodegradation of CLA.

To conclude, the reactive OMPs (DCF, CLA and BTZ) are mainly photodegraded and biodegraded, and their removal can be inhibited by the OMP mixture. The OMPs (MPL, MCPP and CBZ) are hardly bioadsorbed and bioaccumulated.

CRediT authorship contribution statement

Kaiyi Wu: Conceptualization, Data curation, Methodology, Visualization, Writing – original draft. Rosaria Tizzani: Data curation, Methodology, Writing – review & editing. Hans Zweers: Methodology, Writing – reviewing & editing. Huub Rijnaarts: Supervision, Writing – review & editing, Alette Langenhoff: Conceptualization, Visualization, Supervision, Writing – review & editing, Tânia V. Fernandes: Conceptualization, Methodology, Project administration, Supervision, Writing – reviewing & editing.

Declaration of Competing Interest

The authors declare no competing known interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156526.

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