Comprehensive Study on Endocrine Disruptor Removal from Wastewater Using Different Microalgae Species

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Article

Comprehensive Study on Endocrine Disruptor Removal from Wastewater Using Different Microalgae Species

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Abstract: The concentration of endocrine disruptor compounds (EDC) in wastewater is increasing, posing significant risks to living organisms. This study concerns the simultaneous degradation of a variety of EDCs from wastewater, including methylparaben (MeP), propylparaben (PrP), butylparaben (BuP), benzophenone (BP), bisphenol A (BPA) and estrone (E), in the presence of the microalgae *Scenedesmus* sp. or *Chlorella vulgaris*. The potential for abiotic removal of these EDCs and the underlying degradation mechanisms were also studied. The presence of microalgae significantly enhanced the degradation of parabens, achieving complete removal within 7 days, primarily through the mechanism of biodegradation. BPA removal was also improved by microalgae, reaching 82% and 90% within 7 days with *Scenedesmus* sp. and *C. vulgaris*, respectively. BP degradation was predominantly abiotic, accomplishing 95% removal in 7 days. E degradation was mainly abiotic, achieving approximately 40% within 7 days, with a notable contribution from a biodegradation mechanism in the later stages, accounting for 27% and 40% of the final total removal in the presence of *Scenedesmus* sp. and *C. vulgaris*, respectively. This study provides insights into the mechanism of EDC degradation by microalgae, highlighting the potential of *Scenedesmus* sp. and *C. vulgaris* for removing a mixture of EDCs from wastewater.

Keywords: microalgae; endocrine disruptor; nutrients; wastewater treatment

1. Introduction

Endocrine disruptor compounds (EDC) are organic chemicals that interfere with the proper functionality of the endocrine system, causing adverse health effects in organisms and their population [1]. This group of compounds is highly heterogeneous and can be classified in natural or synthetic EDCs. On the one hand, natural EDCs can contain some heavy metals or substances, like phytoestrogens and cyanotoxins, derived from living beings such as plants or bacteria [2]. On the other hand, synthetic EDCs are artificial compounds mostly used for industrial or agricultural purposes, such as pesticides, personal care products (PCPs) or plasticisers [3]. Nowadays, synthetic EDCs, including parabens, benzophenone and its derivatives, bisphenol A, and estrone, are the most common endocrine disruptors due to their extensive production and use [4]. Methylparaben (MeP), propylparaben (PrP), and butylparaben (BuP) belong to p-hydroxybenzoic acid (PHBA) alkyl esters derivates. Parabens are used as preservatives in food, pharmaceuticals, and PCPs due to their antibacterial and antifungal properties [5]. Benzophenone (BP) and its derivatives are the most used UV filters for sunscreen products, skin creams, cosmetics, hair sprays, body lotions, hair dyes, shampoos, and other PCPs [6]. Bisphenol A (BPA) is a diphenylmethane derivative, an organic synthetic compound used as an additive in producing polycarbonate plastics and epoxy resins [7]. Finally, estrone (E) is a naturally occurring hormone belonging to the estrogen family. It is principally

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produced by the ovaries, adipose tissue, fibroblasts, skin, placenta, and brain [8]. The increasing consumption of hormones from contraceptive drugs and hormonal therapy for postmenopausal women is related to elevated levels of estrone in wastewater [9].

It is known that several adverse effects on human health are associated with these synthetic EDCs. For instance, they may increase the proliferation of specific cancer cells [10], disrupt thyroid hormone concentrations, including thyroxine and triiodothyronine [11], and higher urinary levels of these chemicals have been positively correlated with diseases such as diabetes mellitus in adults [12], obesity in children and adolescents [13] or osteoarthritis [14]. Additionally, they may contribute to processes such as feminisation and reproductive dysfunction [15].

Synthetic EDCs are widely distributed throughout the environment, with the highest concentration in water, but also present in air, soil, sludge, and sediments [16–20]. For example, unsubstituted BP was detected in concentrations between 200 and 713 ng/L in two different rivers in Shanghai [6] or BPA can be found within a range of 46.4 and 986 ng/L in different domestic wastewater treatment plants (WWTPs) in Korea [21]. The principal sources of surface water contamination are industrial and urban sewage discharge effluents [22] with conventional WWTPs being inefficient in removing EDCs [23]. For this purpose, various processes have been investigated for removing these EDCs, including adsorption by activated carbon, chemical advanced oxidation, chemical precipitation, or ozonation [24]. However, these treatments have drawbacks, such as low efficiencies when applied to real wastewater or high operational costs [25]. In this context, microalgae provide a promising solution for EDC elimination.

Microalgae are photosynthetic, free-floating microorganisms capable of forming filaments and colonies. They exhibit a remarkable ability to adapt to extreme ecological habitats. Through cellular activities, microalgae convert light and carbon dioxide (CO_2) into various specialised chemicals, including carbohydrates, proteins, lipids, vitamins, and pigments [26]. Compared to previous processes, microalgae-based removal of EDCs offers distinct advantages, including the ability to fix CO_2 [27] and nutrient removal from contaminated water [28]. Moreover, the grown biomass can be further processed for other uses, such as energy production [29]. The removal of EDCs using photosynthetic microorganisms can be driven through four main mechanisms: bioadsorption, bioaccumulation, biodegradation, and photodegradation [30]. The existing literature includes several examples demonstrating the effectiveness of microalgae in removing EDCs. For instance, *Scenedesmus obliquus* and *C. vulgaris* have been shown to achieve removal rates of 99% for BPA, 87% for E, and 100% for both MeP and PrP within 7 days [31]. Additionally, *S. obliquus* biodegraded 96.66 and 74.38% of 0.5 and 2 mg BP-3/L, respectively, in 10 days [32]. Other studies have evaluated the elimination of EDCs using a consortium with different microalgae and cyanobacteria, such as *Anabaena cylindrica*, *Chlorococcus*, *Spirulina platensis*, *Chlorella*, *Scenedesmus quadricauda*, and *Anabaena* sp., achieving approximately 80% removal within 6 days in an algae pond system [33]. Furthermore, *Chlamydomonas mexicana* and *C. vulgaris* can biodegrade 40% of BPA in 10 days [34]. However, all these studies primarily focus on eliminating one or two specific compounds or micropollutants within the same family, often with similar chemical structures. Consequently, there is still a lack of research on the simultaneous removal of mixtures of EDCs from different families using these microorganisms. Thus, the novelty of this work is aligned with this challenge, aiming to provide new insights into the removal and fate of these pollutants using microalgae-based processes.

In this context, *C. vulgaris* and *Scenedesmus* sp. were cultivated to simultaneously remove EDCs (MeP, PrP, BuP, BP, BPA, and E) and nutrients from wastewater. The study included the determination of the main degradation pathways of each EDC (abiotic, bioadsorption, bioaccumulation, and biodegradation).

2. Materials and Methods

2.1. Chemical and reagents

MeP (CAS Number 99-76-3), PrP (CAS Number 94-13-3), BuP (CAS Number 94-26-8), BP (CAS Number 119-61-9), BPA (CAS Number 80-05-7) and E (CAS Number 53-16-7, purity ≥99%) were

 doi:10.20944/preprints202411.1426.v1

purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol LC-MS grade and acetonitrile HPLC grade were acquired from Scharlau (Barcelona, Spain). Mili-Q and distilled water were produced in Autwomatic plus 1+2 (Wasserlab. Barbatáin, Spain). Salts (NaCl, $CaCl_2$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and NH_4Cl) used in synthetic wastewater (SWW) preparation were acquired from Sigma-Aldrich (St Louis, MO, USA). Stock solutions for each EDC were prepared in methanol (1000 mg/L) and stored in darkness in the refrigerator at 4°C. More diluted solutions of 100 and 10 ppm containing all contaminants were prepared in SWW from stock solutions and kept also refrigerated.

2.2. Experiments

EDCs biodegradation was evaluated in the presence of microalgae, light, and aeration (LOM). On the other hand, abiotic removal of EDCs was assessed through control experiments conducted using light (L), aeration (O), and their combination (LO). All experiments were performed in 50 ml batch reactors, with a working volume of 48 mL, over 7 days.

2.2.1. LOM experiments

C. vulgaris and *Scenedesmus* sp. were the selected microalgae species, and the inoculums were acquired from the strain collection of Banco Español de Algas (Las Palmas de Gran Canarias, Spain). The SWW used for microalgae cultivation was prepared according to [35]: 7 mg NaCl, 4 mg $CaCl_2$, 2 mg $MgSO_4 \cdot 7H_2O$, 15 mg KH_2PO_4, 115.6 mg NH_4Cl per liter of deionized water. Reactors in LOM experiments were loaded with a combination of SWW and microalgae inoculum to achieve an initial biomass concentration of 0.1 g/L. Every reactor was additionally spiked with one ppm of each EDC stock solution. Control experiments without contaminants were also performed. Before reaching cultures, all experiments were developed under continuous aeration using air pumps and air filtering using 0.45 µm nylon membranes (Agilent Technologies. Santa Clara, CA, USA). Reactors were additionally irradiated using 12 V LED strips, offering an irradiance of 108 $\mu mol \cdot foton \cdot m^{-2} \cdot s^{-1}$. The photoperiod was established as 12 hours of light followed by 12 hours of darkness. Biomass growth was monitored daily. Dry cell weight (DCW) was determined by filtering 12 mL of microalgae culture through a pre-weighed nylon membrane (GVS. Bologna, Italy) and drying it at 56°C for one week. The dried filters were weighed, and DCW was calculated using weight balance. Additionally, pH was measured daily using a pH meter (XS Instruments. Modena, Italy).

2.2.2. Abiotic experiments

Reactors for L, O, and LO experiments were filled with SWW and spiked with 4.8 mL of 10 ppm stock solution. Experiments L and LO were carried out under light exposure, following the procedure used for LOM runs. Conversely, O experiments were conducted under dark conditions with reactors wrapped with aluminium foil and kept without light. O and LO experiments were conducted under the same aeration conditions as LOM assays.

2.3. Supernatant analysis

The concentration of EDCs in the supernatant was analysed daily for all samples using high-performance liquid chromatography (HPLC) (Agilent Technologies) equipped with a DAD detector (Agilent Technologies, model 1260 DAD WR) and an Ascentis Express C18 column (100 mm x 4.6 mm, 5 µm. Supelco, Merck. Darmstadt, Germany). The mobile phase consisted of acetonitrile (with 0.1% formic acid) and water in a ratio of 30:70 for MeP and 40:60 for the rest of EDCs detection. The flow rate was set to 1 mL/min. Detection wavelengths were established at 254 nm for parabens and BP and 210 nm for BPA and E. The signal-to-noise method was used to determine the limit of detection (LOD) and limit of quantification (LOQ). A signal-noise (S/N) ratio of three was adopted for LOD determination, and an S/N ratio of ten was selected for LOQ [36]. Results are shown in supplementary material (Table S1).

 doi:10.20944/preprints202411.1426.v1

2.3.1. LOM experiments

Samples from LOM experiments were centrifuged at 3500 rpm for 20 minutes. The supernatant was separated from the microalga cell pellets and filtered using a 0.45 μm nylon membrane. Samples were concentrated with solid-phase extraction (SPE), using 500 mg Extrabond C18 cartridges (Scharlau. Barcelona, Spain), sequentially preconditioned with methanol and water. Analytes were finally eluted with 2 mL of methanol, and 10 μL of the eluent was injected for the HPLC analysis previously mentioned. The concentration of ammonia and phosphates were determined with 100683 and 100798 Spectroquant commercial kits, respectively (Merck. Darmstadt, Germany).

2.3.2. Abiotic experiments

Supernatants from L, O, and LO experiments were filtered through a 0.45 μm nylon membrane, and subsequently, EDCs were extracted and analysed by HPLC, as described for LOM experiments.

2.4. Mass balance

A comprehensive study on the removal mechanisms of EDCs by microalgae was conducted to assess the contribution of bioadsorption, bioaccumulation, and biodegradation pathways. The biodegradation percentage of each EDC can be calculated as:

$$P(\%) = (A_t - A_r - A_d - A_a - A_c) \cdot \frac{100}{A_t} \quad (1)$$

Where A_t is the initial amount of each EDC added to the medium (μg at day 0). A_r is the amount of EDC in the supernatant in the LOM experiments. A_d is the amount of EDC adsorbed on the microalgal cell wall. This was calculated by harvesting microalga cells from supernatant in LOM experiments, washing with 1 mL of water, and centrifuging at 3500 rpm for 20 minutes (model 5810, Eppendorf. Hamburg, Germany). The washing water was discarded, and cell pellets were resuspended in 1 mL of methanol. Finally, the pellets were sonicated for 15 minutes (37 kHz, 820 W) with an Elmasonic P bath (Elma Schmidbauer. Singen, Germany) to release EDCs adsorbed on the cell wall. Then, the mixture was centrifuged again, and the adsorbed fraction was quantified, measuring EDC concentration in the solution by HPLC. Finally, A_c is the amount of EDC bioaccumulated inside the microalgal cells. To determine this value, 1 mL of methanol was added to the cell pellet after centrifugation, and the solution was kept under stirring at 900 rpm overnight. To ensure the cell wall lysis, samples were sonicated for 1 hour and then centrifuged at 3500 rpm for 20 min. The concentration of EDCs bioaccumulated by microalgae was determined from the resulting supernatant using HPLC. Finally, A_a is the amount of EDC removed by abiotic mechanisms. The results of the supernatant analysis in the LO experiments were used to estimate the removal fraction of EDCs by abiotic processes.

The kinetic constants for MeP, PrP, BuP, BP, BPA and E removal were also calculated for O, LO and LOM experiments using a pseudo-first order model as follows:

$$LnC_t = -kt + LnC_0 \quad (2)$$

Where C_0 is the initial concentration (ppb) of each pollutant at day 0, C_t is the EDC concentration (ppb) at time t, k is the removal rate constant (h^{-1}), and t is the reaction time (h).

3. Results and discussions

3.1. Microalgae growth in the presence of EDCs

Scenedesmus sp. and *C. vulgaris* were grown separately in the presence (doped) or absence (control) of EDCs mix for 7 days in SWW (LOM experiments), and DCW was evaluated. Figure 1 (a) shows how both microalgae species can grow in culture media with ammonia as a nitrogen source.

 doi:10.20944/preprints202411.1426.v1

C. vulgaris demonstrated superior adaptation to SWW compared to *Scenedesmus* sp., resulting in an algal density of 0.55 and 0.67 g/L within 7 days for control and doped samples, respectively, whereas *Scenedesmus* sp. reached only 0.34 g/L and 0.29 g/L. *C. vulgaris* prefers ammonia as the optimal nitrogen source, even when both ammonia and nitrate are present in the culture media [37]. In contrast, *Scenedesmus* sp. exhibits lower algal density when ammonium is used as a nitrogen source, compared to nitrate or urea [38]. Similar growth for both species was observed in the absence and presence of EDCs. A paired samples t-test revealed that the presence of EDCs did not significantly impact microalgae growth (p-value > 0.05), except on day 1 for both species and day 7 for *C. vulgaris* (p-value < 0.05), where EDCs presence enhanced growth. This fact has also been reported in previous studies, suggesting that microalgae growth is not negatively affected by the presence of EDCs. Instead, microalgae may adapt to low doses of these compounds in culture media, potentially enhancing their growth. For instance, the microalgae *Tetraselmis* sp. demonstrated adaptation to EDCs in the culture media with one ppm of BPA and other EDCs, showing no adverse effects on cell growth [39]. Additionally, the growth of *C. vulgaris* could be enhanced in the presence of MeP concentrations ranging from 1 to 5 ppm [40].

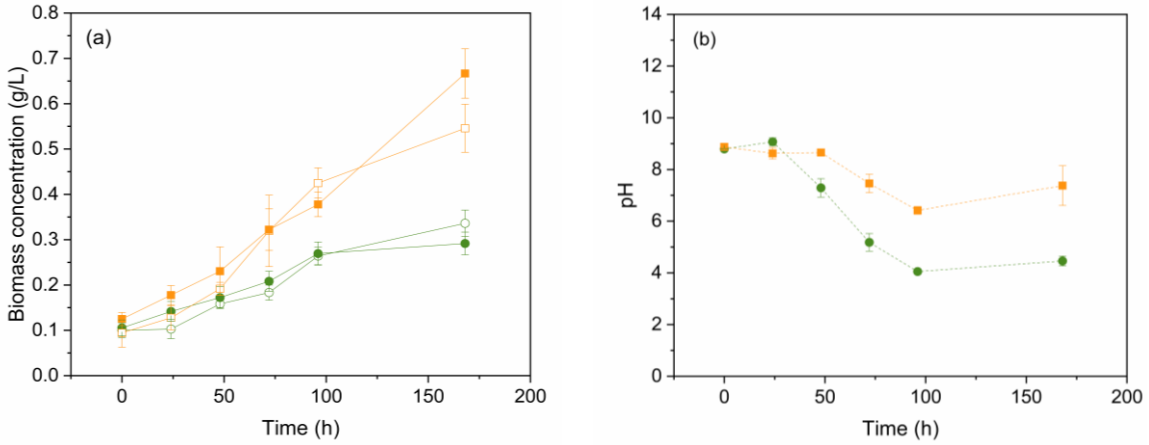

Figure 1. (**a**) Growth curves during 7 days for *Scenedesmus* sp. in the presence (doped – solid green circle) or absence (control – open green circle) of EDCs and *C. vulgaris* in the presence (doped – solid orange square) or absence (control – open orange square) of EDCs. **(b)** pH curves over 7 days for doped *Scenedesmus* sp. (circle, dash green line) and *C. vulgaris* (square, dash orange line). Error bars represent ± standard error of the mean (n=3).

As observed in Figure 1 (b), the pH of *Scenedesmus* sp. culture in the presence of EDCs continuously decays up to a pH value of approximately four after seven days. These acid conditions can be attributed to the release of H^+ when ammonia is the nitrogen source [41]. The optimal pH range for *Scenedesmus* sp. is between 8.5 and 6.5, achieved on day 3 (Figure 1 (b)), which coincides with a decrease in biomass growth, as shown in Figure 1 (a) [42]. Other authors observed this fact, suggesting that the reduction in pH has an inhibitory effect on *Scenedesmus* sp. growth. This inhibition is primarily due to acidic conditions acting as enzyme photosynthesis inhibitors. Consequently, the disruption of photosystem II reactions results in a lower algal cell density [43].

The optimal pH for *C. vulgaris* ranged between 6.0 and 9.0 [44]. Figure 1 (b) shows a slight acidification trend for *C. vulgaris* culture up to a pH of 7. The highest growth rates were observed from day 4 to day 7, as depicted in Figure 1 (a). This increase in algal growth led to elevated algal photosynthetic activity. Some studies suggest that higher photosynthesis rates can lead to a rise in pH, thus preventing the acidification of the culture media [45]. This phenomenon, therefore, is observed in the growth of *C. vulgaris* in the presence of EDCs and explains the high growth rate achieved with this microalga.

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3.2. Nutrient removal

Figure 2 shows ammonia and phosphate removal by *Scenedesmus* sp. (a) and *C. vulgaris* (b) in the presence of EDCs for 7 days (LOM experiments). Figure 2 (a) exhibits a complete phosphate depletion within 4 days of *Scenedesmus* sp. cultivation. Still, only 32% of ammonia was removed in 7 days. This low ammonia removal efficiency is associated with this microalga-limited growth. Thus, the simultaneous complete removal of phosphate restricts ammonia consumption, which has also been reported in previous studies. For example, *Scenedesmus* sp. LX1 completely removes (100%) of total phosphorus (TP) regardless of the N/P ratio, while the removal of total nitrogen (TN) strongly depends on that nutrient ratio [46]. A similar trend was observed when *Scenedesmus* sp. was cultivated under phosphorous-starved conditions. In this study, complete phosphorus depletion occurred within 11 days, but nitrogen removal remained negligible until that point [47].

Figure 2 (b) shows complete ammonia and phosphate removal in 7 days by *C. vulgaris*. This result agrees well with other reports in the literature. In this sense, *C. sorokiniana* completely depleted both ammonia and phosphorus in 7 days when the initial nitrogen concentration ranged from 20 to 40 mg N/L [48]. In addition, *C. vulgaris* cultivated in different anaerobic digestion effluents exhibited nearly 100% removal of ammonia and phosphates within 10 days, with an initial ammonia concentration of 40 mg N/L [49]. The total nutrient removal achieved during the cultivation of *C. vulgaris* in the presence of EDCs provides promising insights for the future development of WWTPs biological treatments based on microalgae.

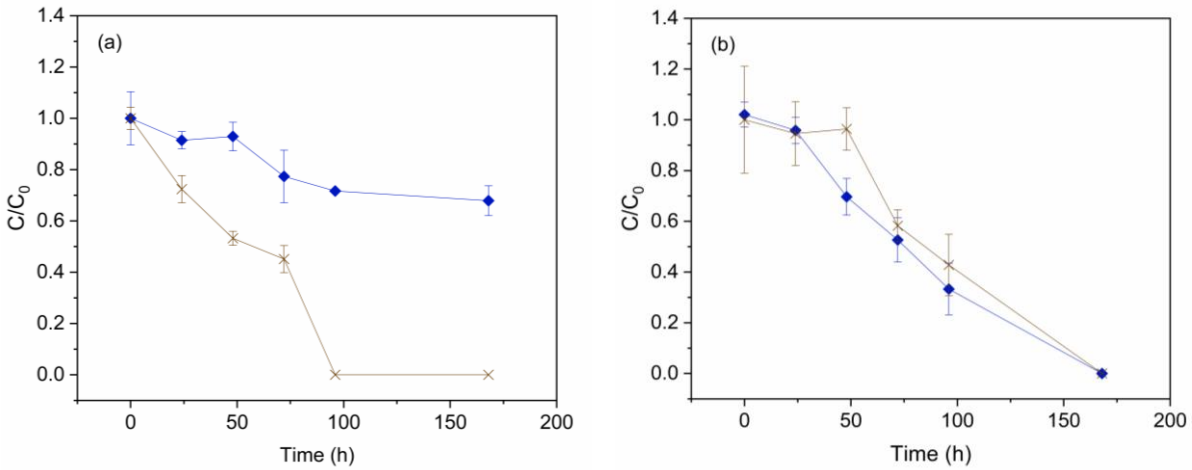

Figure 2. Phosphate (brown cross) and ammonia (blue diamond) removal by **(a)** *Scenedesmus* sp. and **(b)** *C. vulgaris* in the presence of EDCs. Error bars represent ± standard error of the mean (n=3).

3.3. EDCs removal

The influence of light, oxygen, and the presence of microalgae on EDC removal rates was studied for each contaminant. As shown in Figure 3 (a), (b), and (c), parabens do not exhibit a representative abiotic degradation (LO and O experiments). However, the presence of microalgae enhanced removal rates for these compounds, resulting in complete elimination within 7 days for both microalga species, exhibiting excellent performance to degrade these contaminants. These results agree with those observed by other authors. In this sense, high MeP degradation efficiency using *C. vulgaris* was reported, achieving the removal of 1 ppm of MeP within 2 days [40]. Furthermore, complete removal of MeP and PrP, starting at 47.4 and 3.8 ng/L, respectively, was achieved within 7 days using *C. reinhardtii*, *S. obliquus*, *C. pyrenoidosa*, and *C. vulgaris*, separately [31].

Regarding BP degradation, similar high removal efficiencies were observed regardless of the type of experiment, as shown in Figure 3 (d). Thus, the efficiencies of BP removal in the LOM experiments on day 7 were 86% and 62% for *Scenedesmus* sp. and *C. vulgaris*, respectively, slightly lower than the ones obtained in the O and LO experiments (91% and 95%, respectively). These results suggest that the primary degradation pathway for this compound was most likely abiotic. No

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differences in removal rates were observed When comparing O and LO experiments. Therefore, light does not significantly affect the elimination of BP, reaching only 20% of removal within 7 days, as demonstrated in the supplementary material section (Figure S1), which presents the results of the L experiments.

Consequently, BP degradation may involve oxidation due to the presence of oxygen. Previous studies propose that the principal reactive oxygen species (ROS) involved in the photo-transformation of BP–3 are hydroxyl radicals (OH·) and superoxide radicals (·O_2) [50]. In addition, other research hypothesised that BP may be degraded naturally when it is present in water at low concentration [51].

BPA degradation is shown in Figure 3 (e). This contaminant was removed in abiotic conditions (O and LO experiments) by 20%, which agrees with the results previously reported [34]. In addition, the photodegradation (L experiment) of BPA is negligible, as depicted in Figure S1 (Supplementary Material). Conversely, *Scenedesmus* sp. and *C. vulgaris* (LOM experiments) enhance BPA elimination up to 82% and 90% within 7 days, respectively. These results are aligned with previous studies, such as the removal rate of 96% for BPA within 90 hours using a consortium of Chlorophyceae class microalgae and cyanobacteria, using a starting initial BPA concentration of 10 ppb [52]. On the other hand, Li et al. (2009) reported a degradation of BPA nearly 92% within 16 days using *Stephanodiscus hantzschii* in a medium containing one ppm of this contaminant [53].

When analysing E removal, similar degradation values to those obtained for BPA were obtained (by 20%) in abiotic conditions (O and LO experiments) during the first three days, as depicted in Figure 3 (f). However, abiotic degradation became more significant from day 4 to 7. As shown in Figure S1 (Supplementary Material), light was not a significant factor in E removal. The potential synergistic effects between these pollutants were studied since BP and E were the only compounds that exhibited abiotic degradation. For this purpose, LO experiments were conducted without BP and E, keeping the operating conditions and the rest of the ECDs. Results indicated that E did not undergo abiotic degradation under LO conditions without B (Supplementary material, Figure S2 (b)). However, BP exhibited a similar degradation trend in the culture media with or without E, with the degradation rate being even higher in the presence of E at day 7 (95% compared to 60%, respectively) (Supplementary material, Figure S2 (a)). These findings suggest a positive synergistic effect on the degradation of BP and E when both compounds are present in the mixture. On the other hand, when *C. vulgaris* was included, a dramatic increase in removal efficiency was observed from day 4 to day 7, achieving a reduction of E by 83% within 7 days (Figure 3(f)).

Conversely, the presence of *Scenedesmus* sp. did not increase the percentage of E removed (70% in 7 days) compared to O experiments, but it was higher than in the LO experiments. During their growth, algae can produce algal extracellular organic matter (AEOM), mainly composed of biopolymers such as polysaccharides and proteinaceous substances. AEOM may promote the photochemical production of short-lived radicals, including excited triplet state dissolved organic matter (^{3}DOM*), singlet oxygen ($^1O_2$), and OH·, which can contribute to the degradation processes of pollutants [54]. Previous studies suggest that *C. vulgaris* produces more AEOM than *Scenedesmus quadricauda*, potentially leading to more efficient photocatalytic degradation of estrogens [55]. Consequently, the high E removal values achieved with *C. vulgaris* from day 4 to 7 (Figure 3 (f)) coincide with the fastest growth rate during the exponential phase for this species (Fig. 1 (a)), a period characterised by the highest metabolic activity in which AEOM release increases [54].

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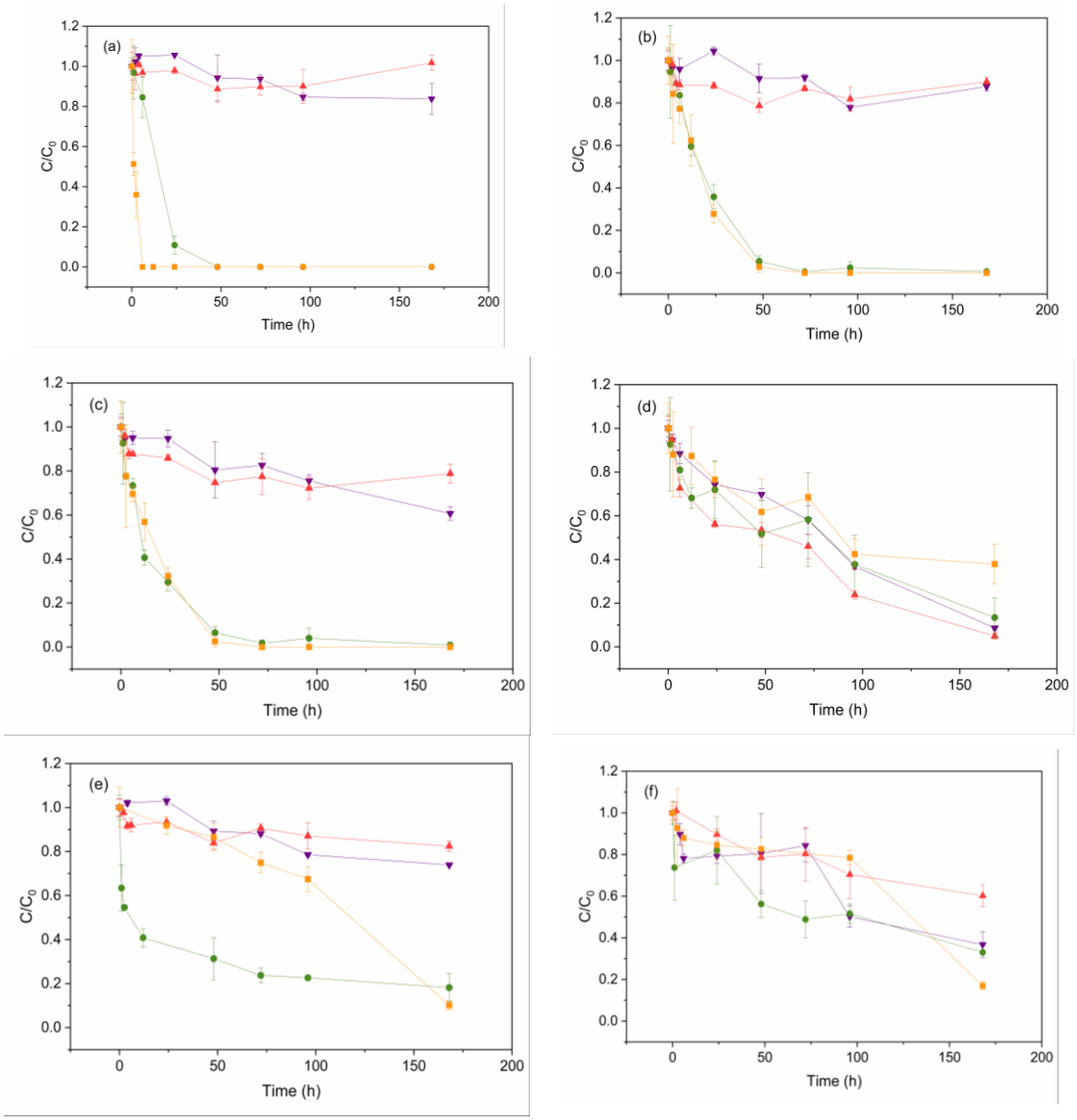

Figure 3. EDC removal: O experiments (purple down triangle), LO experiments (red triangle), and LOM experiments with *Scenedesmus* sp. (green circle) or with *C. vulgaris* (orange square): **(a)** MeP, **(b)** PrP, **(c)** BuP, **(d)** BP **(e)** BPA and **(f)** E removal. Experiments were conducted for 7 days. Error bars represent ± standard error of the mean (n=3).

The differences in AEOM release could explain the variation in final E removal between *C. vulgaris* and *Scenedesmus* sp. These removal results are aligned with previous research. For instance, a 79% reduction in E concentration was reported using *Haematococcus pluvialis*, *Selenastrum capricornutum*, and *Scenedesmus quadricauda* individually over 10 days [56]. Additionally, an overall E removal of 91% for *S. obliquus* and 52% for *C. vulgaris* in 5 days was demonstrated [57]. In this study, *Scenedesmus* achieved a higher removal efficiency compared to *C. vulgaris*, which contrasts with our findings. This discrepancy could be attributed to differences in cultivation methods and the composition of the synthetic wastewater used. For instance, they employed a continuous cultivation mode, where reactors were fed daily, providing a more stable environment and consistent nutrient supply, potentially enhancing the performance of *Scenedesmus* over *C. vulgaris*.

Finally, the final removal percentages obtained for both microalgae after 7 days are presented in Table 1 and compared with previous literature. As explained previously, comparing these results with others reported in the literature is difficult due to the considerable influence of culture conditions, such as the presence of aeration and the composition of the culture media.

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Table 1. Final removal percentage rates at day 7 with *Scenedesmus* sp. and *C. vulgaris* and comparison with literature review. Errors are expressed as ± standard error of the mean (n=3).

Microalgae	MeP	PrP	BuP	BP	BPA	E	Ref.
Scenedesmus sp.	100.0±0.0%	99.4±1.1%	99.2±1.5%	85.6±9.0%	81.8±6.3%	67.0±0.6%	This study
C. vulgaris	100.0±0.0%	100.0±0.0%	100.0±0.0%	62.1±9.1%	89.9±2.0%	83.0±2.0%	
C. reinhardtii	100.0%	100.0%					[31]
S. obliquus	100.0%	100.0%					
C. pyrenoidosa	100.0%	100.0%					
C. vulgaris	100.0%	100.0%					
Tetradesmus obliquus, C. vulgaris, Pseudanabaena sp., *Scenedesmus* sp. *and Nitzscha* sp.		89.0%					[58]
C. vulgaris	33.0-14.0%						[59]
S. obliquus				23.3–28.5% [1]			[32]
Chlamydomonas reinhardtii				58.4% [1]			[60]
C. vulgaris				14.0% [2]			[61]
Chlorella pyrenoidosa					20.0-43.0%		[62]
C. mexicana					39.0%		[34]
C. vulgaris					28.0%		
S. obliquus						91.0%	[57]
C. vulgaris						52.0%	

[1] BP-3

[2] BP-4

Regarding parabens, complete removal was achieved for both microalgae. These removal percentages are within the range described by other authors. As previously mentioned, 100% elimination of MeP and PrP using different microalgae species was reported [31]. Additionally, a PrP removal rate of approximately 89% was achieved from an initial concentration of 300 ng PrP/L, using an anoxic-aerobic photobioreactor with a consortium of *Tetradesmus obliquus*, *C. vulgaris*, *Pseudanabaena* sp., *Scenedesmus* sp., and *Nitzscha* sp. [58]. However, these studies employed lower initial concentrations than those reported in our work. When higher concentrations of parabens were studied, only 33% and 14% of MeP removal were obtained using *C. vulgaris* at initial concentrations of 0.8 and 8.0 mg MeP/L, respectively [59]. Given the scarcity of literature on BP microalgae removal, our results will be compared with those for similar molecules such as BP-3 or BP-4. In all cases, the removal values obtained in our study are superior to those reported in previous research. *S. obliquus* removed 23.3–28.5% of BP-3 after 10 days of cultivation, ranging from 0.1 to 3 mg BP-3/L [32]. A maximum BP-3 removal of 58.4% was achieved at an initial concentration of 0.01 μg BP-3/L using the green alga *Chlamydomonas reinhardtii* within 10 days, decreasing efficiency at higher pollutant concentration [60]. A removal rate of 14% at 1 mg BP-4/L was reported also using *C. vulgaris* [61]. The final BPA removal rates presented in Table 1 are higher than those reported by previous authors. Removal rates of 20.0%, 46.4%, 42.9%, and 43.0% were achieved using *Chlorella pyrenoidosa* after 120 h of culture with BPA concentrations of 2.0, 4.0, 6.0 and 8.0 mg BPA/L [62]. Microalgae *C. mexicana* and *C. vulgaris* achieved 39% and 28% removal, respectively, in a medium containing 1 mg BPA/L within 10 days [34]. Finally, the results of E removal are consistent with the previously shown literature. *S. obliquus* and *C. vulgaris* removed 91% and 52% of E, respectively [57], and other studies found E removal rates higher than 80% [56]. In summary, the results reported in our work are very promising and demonstrate the potential of using *Scenedesmus* sp. and *C. vulgaris* to simultaneously remove a variety of EDCs from wastewater, achieving ECD removal percentages, in general, higher than those reported in the literature.

To quantify degradation rates for each contaminant, kinetic constants were calculated from the above removal curves, assuming a pseudo-first-order model in all cases, according to equation (2). The values obtained (Table 2) indicate that the degradation rate is significantly higher when using microalgae compared to abiotic conditions, with *C. vulgaris* being superior to *Scenedesmus* sp. for

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parabens removal. For BP, the highest degradation rates were achieved under abiotic conditions, as explained above, reinforcing the hypothesis that the primary degradation pathway for this contaminant is driven in these conditions. Regarding BPA degradation, Table 2 shows that the rate constant values are higher when microalgae are present. This, along with the high removal efficiency values mentioned earlier, suggests the suitability of these species to degrade this compound. Finally, in the case of E removal, the rate constants were similar, obtaining higher values in the presence of microalgae.

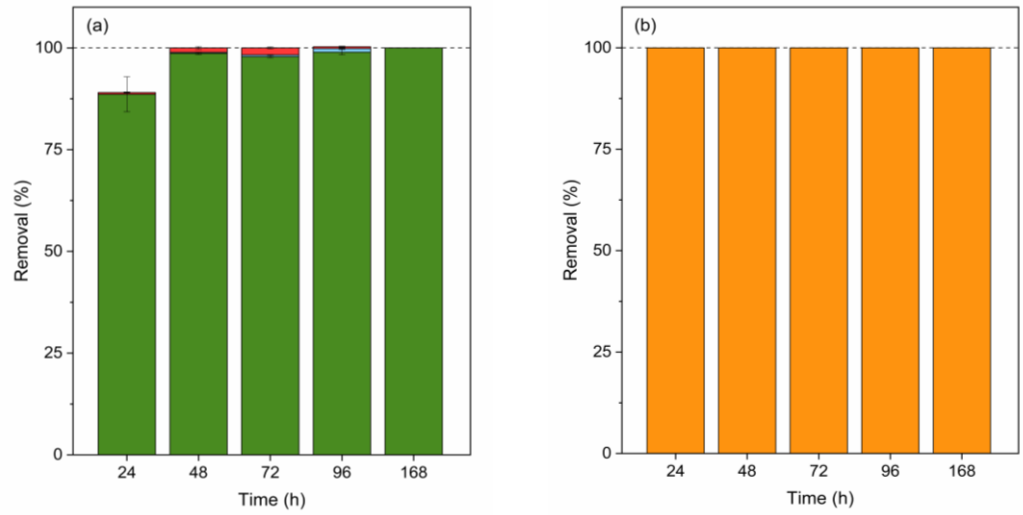
Table 2. Removal rate constants (k) estimated for abiotic EDC degradation and biodegradation using first-order kinetics.

Compound	k_O (h^{-1}) (R^2)	k_{LO} (h^{-1}) (R^2)	k_{LOM} (h^{-1}) (R^2)	
			Scenedesmus sp.	*C. vulgaris*
MeP	$1.3 \cdot 10^{-3}$ (0.868)	$1.1 \cdot 10^{-3}$ (0.841)	$9.0 \cdot 10^{-2}$ (0.983)	$3.8 \cdot 10^{-1}$ (0.928)
PrP	$7.0 \cdot 10^{-3}$ (0.831)	$4.0 \cdot 10^{-3}$ (0.758)	$5.7 \cdot 10^{-2}$ (0.968)	$7.4 \cdot 10^{-2}$ (0.972)
BuP	$2.9 \cdot 10^{-3}$ (0.951)	$2.8 \cdot 10^{-3}$ (0.961)	$5.4 \cdot 10^{-2}$ (0.982)	$7.5 \cdot 10^{-2}$ (0.957)
BP	$1.3 \cdot 10^{-2}$ (0.936)	$1.6 \cdot 10^{-2}$ (0.958)	$1.1 \cdot 10^{-2}$ (0.924)	$6.0 \cdot 10^{-3}$ (0.901)
BPA	$2.0 \cdot 10^{-3}$ (0.940)	$1.1 \cdot 10^{-3}$ (0.956)	$2.1 \cdot 10^{-2}$ (0.976)	$1.3 \cdot 10^{-2}$ (0.815)
E	$5.9 \cdot 10^{-3}$ (0.966)	$3.1 \cdot 10^{-3}$ (0.954)	$5.2 \cdot 10^{-3}$ (0.819)	$9.9 \cdot 10^{-3}$ (0.805)

3.4. Mass balance

Figures 4 to 7 depict the contribution of the different removal mechanisms (abiotic, biosorption, bioaccumulation, and biodegradation) for the studied contaminants within 7 days. The results were calculated using equation (1) and expressed as the amount of contaminant removed with respect to its initial concentration for each experiment.

The primary removal mechanism for parabens in both species is biodegradation (Figure 4 (a), (b), (c), (d) and (f)). However, *Scenedesmus* sp. also showed a low percentage of biosorption and bioaccumulation of MeP (Figure 4 (a)) and PrP (Figure 4 (c)). The adsorbed fraction exhibited a consistent trend for these compounds, initially increasing from day 1 to day 3 (from 0.43 to 1.71% for MeP and from 0.57 to 1.44% for PrP), and subsequently decreasing until day 7, reaching less than 0.11% for MeP and 0.64% for PrP. On the other hand, the bioaccumulated fraction of MeP increased during the initial days from 0.04-0.11% to 0.88% but showed an almost complete reduction (<0.11%) by day 7. Conversely, the bioaccumulated fraction of PrP showed an upward trend from day 1 to day 7, increasing from less than 0.005% to 0.58%. These results suggest that MeP and PrP presented a slow diffusion into the cells, and probably MeP underwent biodegradation by intracellular enzymes. The adsorbed and bioaccumulated fractions for BuP (Figure 4 (e)) were negligible, accounting for less than 0.08% of the total contaminant concentration removed. The concentrations detected in both measurements were below the LOD.



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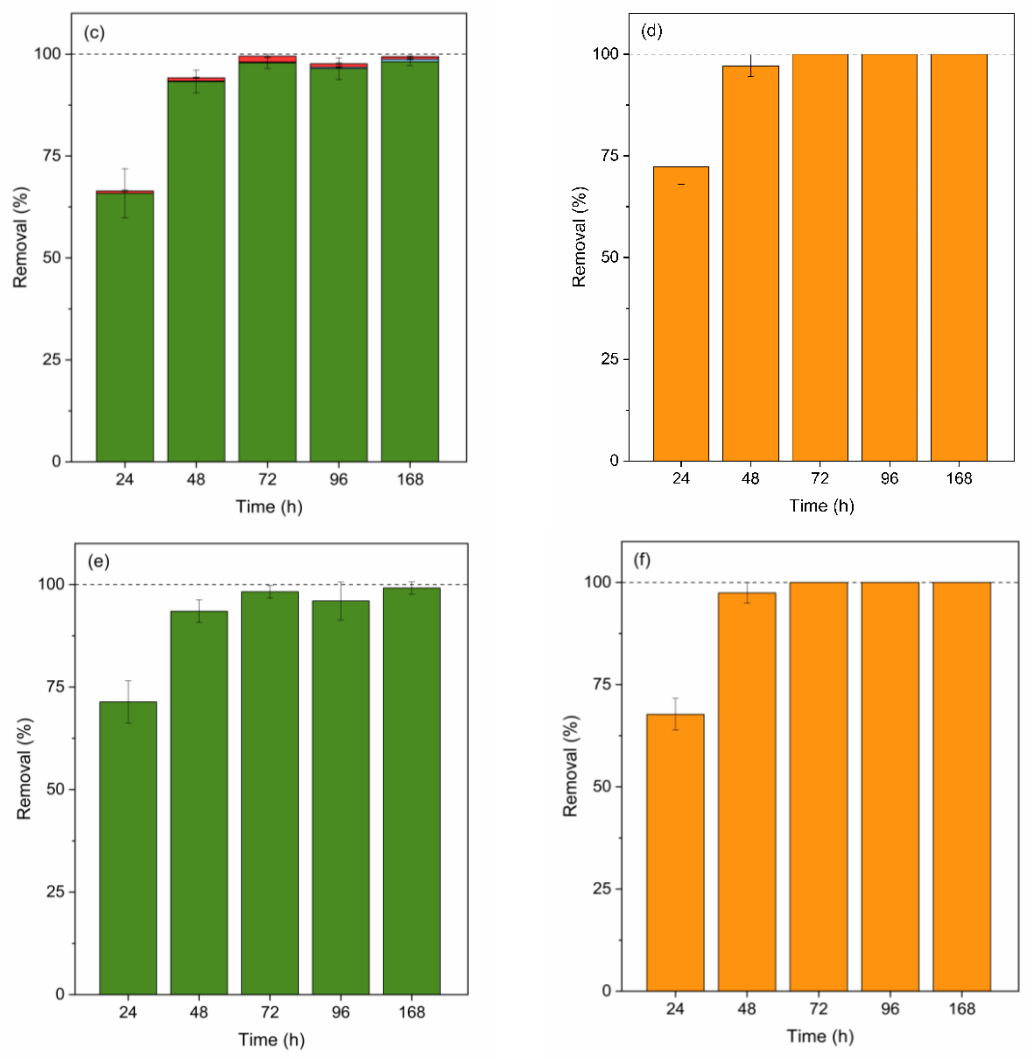

Figure 4. Contribution (%) of abiotic degradation (yellow), biosorption (red), bioaccumulation (blue), and biodegradation (green *Scenedesmus* sp. and orange *C. vulgaris*) in MeP, PrP, and BuP removal by *Scenedesmus* sp. **(a, c and e)** and *C. vulgaris* **(b, d, and f)**. Error bars represent ± standard error of the mean (n=3).

The absence of parabens adsorbed by this species can be observed by analysing paraben removal results for *C. vulgaris*. In this sense, previous studies reported that MeP biosorption on this species is negligible due to the hydrophobicity of its cell wall [59]. This fact agrees with our results, which showed adsorbed fractions lower than 0.05% for MeP and PrP and ranged between 0.53 and 0.11% for BuP. Furthermore, the contribution of the bioaccumulation pathway to the removal of the studied parabens was also negligible. When EDCs are present inside microalgae cells, an oxidative stress defence mechanism may be triggered, leading to the formation of ROS in organelles. According to the literature, no excess ROS formation was observed during the growth of *C. vulgaris* in the presence of 0.8 mg MeP/L over 7 days, reinforcing the theory that parabens do not diffuse into the cells of this species [59]. Thus, biodegradation appeared as the main pathway for parabens degradation in *C. vulgaris*.

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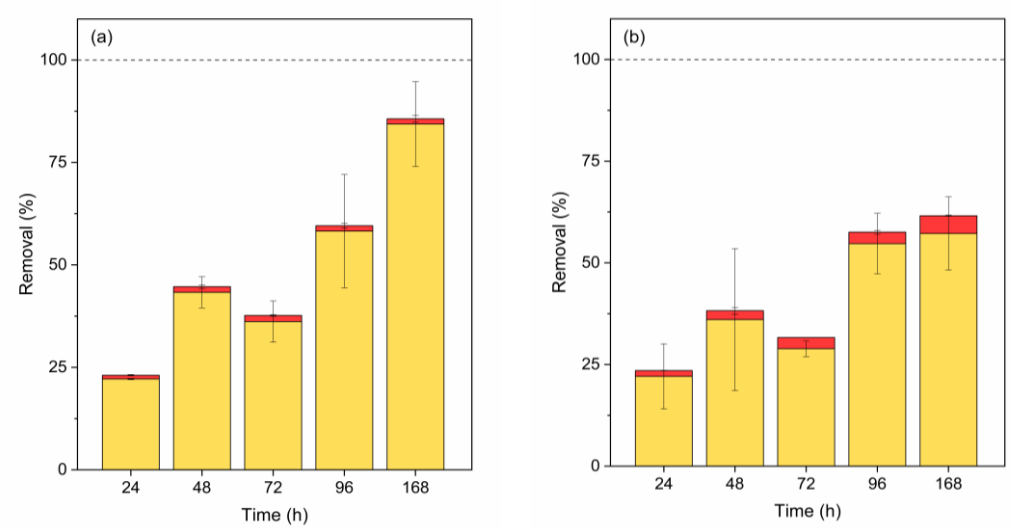

Figure 5. Contribution (%) of abiotic degradation (yellow), biosorption (red), bioaccumulation (blue), and biodegradation (green *Scenedesmus* sp. and orange *C. vulgaris*) in BP removal by *Scenedesmus* sp. **(a)** and *C. vulgaris* **(b)**. Error bars represent ± standard error of the mean (n=3).

BP exhibited a remarkable abiotic degradation pathway (Figure 5 (a) and (b)). As previously explained, no significant differences were observed between removal efficiency in LO experiments (abiotic conditions) for BP regardless of the microalgae used, obtaining a p-value > 0.05 in both cases. Besides, BP showed low adsorption onto the cell wall, being this fraction higher when using *C. vulgaris* (Figure 5 (b)) compared to *Scenedesmus* sp. (Figure 5 (a)). As can be observed in Figures 5 (a) and 5 (b), the contribution of the bioaccumulation pathway was negligible for both microalgae species. In the case of *Scenedesmus* sp., values ranging between 0.09 and 0.52% (concentrations between LQD and LOD) were obtained from day 1 to day 7. A similar trend was observed for *C. vulgaris*, ranging bioaccumulation percentages of BP between less than 0.12% (concentrations below the LOD) and 0.53% from day 1 to 7, respectively. Comparing the obtained results with previous studies on unsubstituted BP is challenging due to the scarcity of such studies. The most similar compounds found in the literature are BP-3 and BP-4. In this way, Lee et al. (2020) achieved 97% biodegradation of 0.5 ppm of BP-3 within 10 days using *S. obliquus*, and approximately 3% attributed to abiotic removal [32]. Similarly, Huang et al. (2018) found negligible BP-4 adsorption and absorption onto *C. vulgaris* strains, with the concentration decreasing solely due to biodegradation, achieving 14% at one mgBP-4/L within 13 days [61]. The lower abiotic degradation observed in the literature compared to our research is attributed to differences in cultivation conditions. Specifically, their cultures were maintained only with agitation, without aeration, leading to the absence of oxygen-derived ROS in the culture medium, which promotes BP removal.

The main removal pathway for BPA using *Scenedesmus* sp. is biodegradation (Figure 6 (a)). Adsorption accounted for less than 1% of total removal during the two first days, decreasing to less than 0.06%. Additionally, BPA was not detected inside the cell, indicating that the bioaccumulated fraction was negligible, remaining below 0.06% (under LOD). Biosorption and bioaccumulation mainly contributed to BPA removal using *C. vulgaris* (Figure 6(b)). Both pathways were similar during the first three days, with bioaccumulation becoming predominant from day 4 onwards. These results agree with other authors, such as Ben Ouada et al. (2018), who reported abiotic removal of BPA by 19%, with the fraction removed by accumulation, adsorption, and biodegradation by 1.3%, 11.6%, and 40%, respectively, within 5 days using the alkaliphilic Chlorophyta *Picocystis* with an initial concentration of 25 mg BPA/L [63]. -Similarly, abiotic removal values of BPA by 15.0% were found, observing biodegradation pathway contribution by 25% using *C. mexicana* and *C. vulgaris*, respectively, at the end of the 10-day experiment with initial concentrations of 1 mg BPA/L [34].

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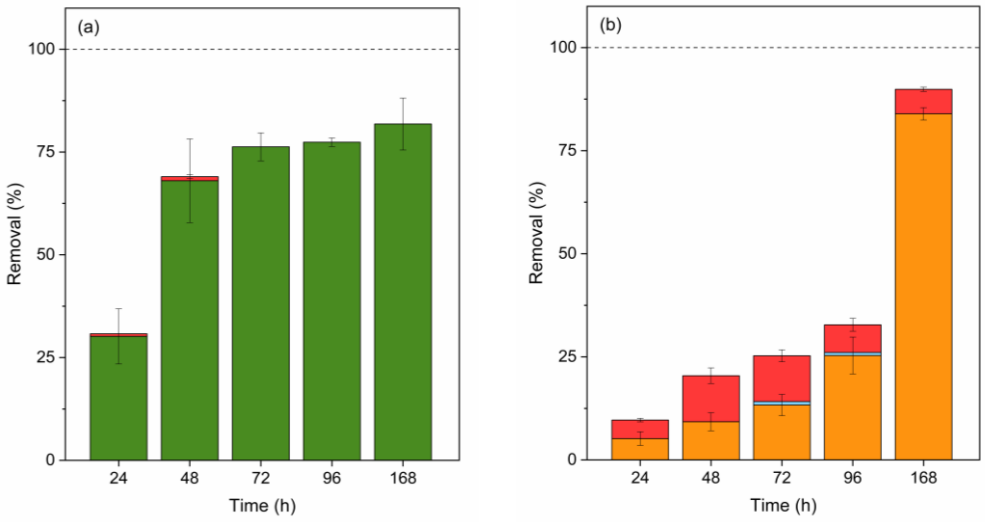

Figure 6. Contribution (%) of abiotic degradation (yellow), biosorption (red), bioaccumulation (blue), and biodegradation (green *Scenedesmus* sp. and orange *C. vulgaris*) in BPA removal by *Scenedesmus* sp. **(a)** and *C. vulgaris* **(b)**. Error bars represent ± standard error of the mean (n=3).

As can be observed in Figures 7 (a) and (b), abiotic degradation and biodegradation are the main pathways to remove E. Comparing *Scenedesmus* sp. (Figure 7 (a)) and *C. vulgaris* (Figure 7 (b)), it can be observed that in the case of the former, E removal is carried out at earlier stages than in the latter. In contrast, *C. vulgaris* only presented a biodegradation contribution on the last day. As previously explained, *C. vulgaris* potentially releases AEOM at the end of the exponential growth phase, which promotes E degradation. Comparing our results is challenging because E is one of the least studied estrogens in this context, with limited previous research studies available. Additionally, among the existing studies, E is often examined as a degradation product of 17β-estradiol. For example, Ruksrithong and Phattarapattamawong (2019) found that adsorption accounted for 10% of total E removal using *S. obliquus* and *C. vulgaris*, identifying biodegradation as the primary removal mechanism for both species. The biodegradation of E by *S. obliquus* was 77%, whereas *C. vulgaris* degraded only 38% within 5 days [57]. As explained earlier, these results are only partially aligned with ours, primarily due to differences in cultivation.

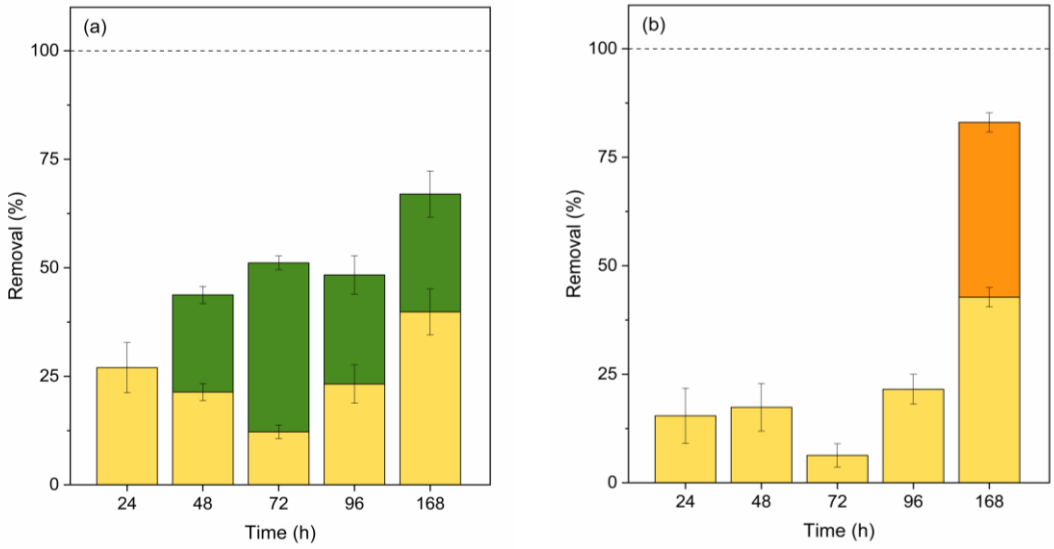

Figure 7. Contribution (%) of abiotic degradation (yellow), biosorption (red), bioaccumulation (blue), and biodegradation (green *Scenedesmus* sp. and orange *C. vulgaris*) in E removal by *Scenedesmus* sp. **(a)** and *C. vulgaris* **(b)**. Error bars represent ± standard error of the mean (n=3).

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4. Conclusions

Scenedemus sp. and *C. vulgaris* were used to remove various EDCs. The total removal ratios of parabens (MeP, PrP, and BuP) were higher in the presence of both microalgae, achieving a 100% removal within one or two days. Abiotic removal was not significant in this process, with biodegradation being the primary mechanism, while adsorption and bioaccumulation were negligible. BP and E exhibited high abiotic degradation, likely induced by derived oxygen ROS. However, the biodegradation pathway contributed significantly to E removal for both *Scenedesmus* sp. and *C. vulgaris*, achieving final degradation rates of 67% and 83%, respectively. Biodegradation was the primary degradation pathway for BPA combined with adsorption. The contribution of the latter pathway was higher in the case of *C. vulgaris* than in *Scenedesmus* sp., with final degradation percentages of 90% and 82%, respectively. Overall, the promising results obtained in this work are of interest in the field, as they demonstrate the suitability of *Scenedesmus* sp. and *C. vulgaris* for EDC removal. This was proven not only for a specific family of these contaminants but for a variety of them with different characteristics simultaneously, showing the potential application of these microalgae for EDC removal from wastewater. Besides, the findings reported in this work on the routes implied in EDC removal can be a benchmark for further studies on the metabolic pathways driven by microalgae in these processes. Finally, the results of this research can have future implications in using microalgae for wastewater treatment, serving as a starting point for scaling up these processes.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Analytical parameters obtained for EDCs with HPLC-DAD for suspended fraction. LOD and LOQ; Figure S1: L experiment EDCs removal for MeP (black square), PrP (red circle), BuP (blue triangle), BP (green down triangle), BPA (purple diamond), and E (orange cross). Error bars represent ± standard error of the mean (n=3); Figure S2: BP removal (a) and E removal (b) for LO experiment (red triangle) and LO without E and BP (blue diamond). Error bars represent ± standard error of the mean (n=3).

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