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Insights into removal of antibiotics by selected microalgae (*Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, *Dunaliella tertiolecta* and *Pseudokirchneriella subcapitata*)

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Abstract

The increasing contamination of worldwide water with numerous antibiotics has become an emerging environmental concern due to the considerable ecotoxicity and health issues associated with some antibiotics. Microalgae-mediated remediation of pollutants is of growing scientific interest due to the ability of microalgae to take up organic/inorganic nutrients and produce biomass that can be further used for energy or biofuel generation. Elimination of pollutants from water in microalgae experiments includes various mechanisms, such as photodegradation, sorption in biomass and biodegradation. The

sorption of pollutants by microalgae has been widely reported, but few studies have proven the biodegradation of pollutants by microalgae. In this work, the ability of four selected microalgae (*Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, *Dunaliella tertiolecta* and *Pseudokirchneriella subcapitata*) to remove 9 antibiotics and the antidepressant venlafaxine was studied under different experimental conditions. The results show that the highest efficiency for antibiotic removal was obtained with microalgae exhibiting the highest growth rates. Promising results have been obtained from *C. reinhardtii* and *D. tertiolecta* cultures, which exhibited a rapid growth and good removal percentages for some of the antibiotics studied. Photodegradation was the main removal mechanism for the 3 fluoroquinolones studied (ciprofloxacin, ofloxacin, norfloxacin) and pipemidic acid (>78%), while for the 3 macrolides (azithromycin, clarithromycin, erythromycin), the efficiency of photodegradation was less than 5%. For the macrolides, the combination of abiotic phenomena, sorption onto the biomass and biodegradation were the mechanisms responsible for total removal. Trimethoprim and venlafaxine were shown to be very stable compounds with low removal percentages. Sulfapyridine appeared to be eliminated mainly by algae biodegradation since low sorption and photodegradation efficiencies were observed. The presence of products resulting from transformations of azithromycin, erythromycin and sulfapyridine in the liquid phase of live algae confirmed that biodegradation is one way by which microalgae eliminate these antibiotics.

Keywords:

Microalgae; pharmaceuticals; bioremediation; transformation products

1. Introduction

Pharmaceutically active compounds (PhACs) are emerging contaminants (ECs) that can pose potential direct risks to the environment and indirect risks to human health and are thus a subject of concern from scientists and society. The presence of PhACs in the environment has increased in recent years as a result of the increased consumption of both human and veterinary medicines, and concentrations in the range of ng/L and µg/L [1–4] have been detected in surface and groundwater bodies. Despite their low concentrations in the aquatic environment, they might elicit adverse effects in organisms, including the spread of resistant genes and bacteria, and are thus becoming an issue of particular concern [5–11]. Antibiotics are ingested, excreted as unchanged active compounds but also in the form of metabolites, and finally disposed of in the receiving environment in discharges from wastewater treatment plants (WWTPs) [12]. Conventional wastewater treatment plants (WWTPs) are not totally successful in their removal [13,14], and therefore, there is a need for new wastewater treatment strategies. Physico-chemical treatments (i.e., advanced oxidation processes, activated carbon adsorption or membrane filtration) have already been studied [15–17] and can attain efficient removal of micropollutants in wastewaters, but in some cases, the pollutants are concentrated as waste to be treated. Alternatively, biological methodologies such as membrane bioreactors (MBRs), which use high biomass concentration and sorption on the membrane to improve the elimination of ECs compared to conventional activated sludge treatments [18], or the use of fungal reactors have the capacity to achieve higher removal percentages than conventional treatments [19–21]. In biological processes, identification of transformation products (TPs) is important for demonstrating the biotransformation of pollutants. Among biological treatments, microalgae-based technologies constitute low-energy input systems that have recently been considered for EC removal from wastewater

effluents [22,23]. These systems permit high exposure to light and are usually operated with high hydraulic retention times (HRTs). In addition to the high quality of the treated effluent obtained (exhibiting reasonable levels of micropollutant removal), microalgae WW treatment technologies are also relevant due to the recovery of algal biomass, which is used as a source of different products such as biofuel or fertilizers [24–26].

It is important to understand the mechanisms for degradation and elimination of PhACs by microalgae to know how these compounds will affect the end-use of the algal biomass [27].

Currently, most of the studies regarding the ECs removal capacities of microalgae focus on the removal of few ECs at the laboratory scale, including axenic cultures. The elimination of ECs such as oestrogenic compounds with microalgae has been widely documented [28–30]. Some studies have focused on antibiotic biodegradation with pure microalgae. For example, Zhou et al. [31] employed 4 freshwater green microalgae (*Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Chlorella pyrenoidosa* and *Chlorella vulgaris*) for the removal of nutrients, metals and 50 contaminants, including antibiotics. Experiments were carried out on the batch scale by treating real sterilized WW. The study clearly demonstrated the capability of the four algae to remove (>50% removal) most of the selected organic contaminants in WW. More than 80% efficiency for removal of clarithromycin, roxithromycin and triclocarban was even achieved by the four algae species. Liu et al. [32] reported 35% efficiency for degradation of spiramycin and amoxicillin by the microalgae *Microcystis aeruginosa*. Santaefemia et al. [33] concluded that the microalgae *Phaeodactylum tricomutum* removed 97% of the antibiotic oxytetracycline through a combination of biodegradation and photodegradation processes. Bai and Acharya [34] found that the mechanisms used by the green alga *Nannochloris sp.* for removal of the antibiotic sulfamethoxazole and the bactericide

triclosan were different: photodegradation played a major role in sulfamethoxazole removal, whereas uptake was the primary mechanism for removing triclosan.

Recently, Xie et al. [27] demonstrated that *Chlamydomonas* sp. Tai-03 was capable of efficient removal of ciprofloxacin and sulfadiazine by biodegradation (65-105%) and photolysis (35-60%), respectively. They point out that adsorption may play an essential role in promoting biodegradation, as ciprofloxacin is more readily absorbed onto biomass than sulfadiazine.

Algae play an important role in the indirect photolysis of antibiotics since they act as photosensitizers and promote this reaction by inducing the generation of active species through the excretion of extracellular organic matter. The use of indirect photolysis plays a dominant role in the removal of organic chemicals [35], and the rates are much faster than those of direct photolysis. Tian et al. [36] found that the chlortetracycline removal efficiency was close to 96% in the presence of *Chlorella vulgaris* under LED irradiation. Moreover, Tian et al. [37] reported promotion of the rate for chlortetracycline photodegradation in the presence of the algae *Chlorella vulgaris*, *Microcystis aeruginosa*, and *Scenedesmus*. Likewise, Zhang et al. [38] demonstrated that *C. vulgaris* increases the norfloxacin degradation rate threefold under light compared with the solution without algae.

In considering the use of algae systems for WW treatment, De Godos et al. [39] used a pilot-scale high-rate algal pond (HRAP) fed with synthetic WW for the removal of the antibiotic tetracycline by a combination of sorption and photodegradation mechanisms. Matamoros et al. [40] conducted the first study on the removal of ECs mixtures in two pilot-scale HRAPs by treating urban WW. For triclosan, which is postulated to be a critical compound in terms of contribution to the environmental risk of wastewater effluent, removals of approximately 93-95% in the warm season and approximately 49-

69% in the cold season were obtained. Clarithromycin and roxithromycin were efficiently removed (>80%), whereas lincomycin was poorly removed. The removal mechanisms were identified as photodegradation and biodegradation for most of the ECs studied, results that are in accord with those of other authors [41]. These studies found that algae removed some ECs from water successfully, but the different mechanisms for removal of ECs in algae-mediated processes are not fully understood. Understanding the different mechanisms for removal of ECs by microalgae and identifying the transformation products could provide significant information on the potential use of algae for removal of ECs from wastewater effluents.

The aim of this study was to determine microalgae capacity for the removal of 9 antibiotics (including macrolides, fluoroquinolones, sulfonamides and others) and an antidepressant (venlafaxine) using four different microalgae strains (*Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, *Dunaliella tertiolecta* and *Pseudokirchneriella subcapitata*). Removal mechanisms were evaluated using different experimental conditions, and these results were supported by the identification of selected TPs of some antibiotics.

2. Materials and methods

2.1. Microalgae strains and cultivation conditions

Chlamydomonas reinhardtii (UTEX ID 2243), *Chlorella sorokiniana* (UTEX ID 1663), and *Pseudokirchneriella subcapitata* (UTEX ID 1648) were supplied in agar tubes, whereas *Dunaliella tertiolecta* (UTEX ID LB999) was supplied in liquid media. All of them were from the culture collection at the University of Texas (UTEX).

Experimental cultures were carried out in 250 mL Erlenmeyer flasks under continuous irradiation with a fluorescent lamp ($172 \pm 18 \mu\text{mol}/(\text{m}^2 \cdot \text{s})$ irradiance level), which was

monitored by a light metre (LI.189, LI-COR Quantum/Radiometer/Photometer, USA) at a controlled temperature (25 ± 1 °C) and 120 rpm (orbital shaker Kuhner, LS-X, Switzerland). Microalgae were grown in their appropriate growth medium (GM): *C. reinhardtii* and *C. sorokiniana* were cultured in Tris-acetate-phosphate (TAP) medium, *D. tertiolecta* in artificial sea water and *P. subcapitata* in Bold 3N medium [42].

2.2. Chemicals and reagents

For the batch experiments, azithromycin (AZI), clarithromycin (CTM), erythromycin (ERY), ciprofloxacin (CFC), ofloxacin (OFC), norfloxacin (NFC), trimethoprim (TMP), sulfapyridine (SPY) and pipemidic acid (PMA) were analytical grade and purchased from Sigma-Aldrich (Barcelona, Spain). Venlafaxine (VFX) was from USP (United States Pharmacopeial Convention) and supplied from Cymit Quimica (Barcelona, Spain).

Target pollutants were dissolved in dimethyl sulfoxide (DMSO) and a solution of 0.1 mg/L of each was prepared. All other reagents were analytical grade from Sigma-Aldrich (Barcelona, Spain).

For the chemical analysis, all PhACs standards included in this study were obtained at high purity grade (>95%). Internal standards ofloxacin-d₃, erythromycin-N,N¹³C and ciprofloxacin-d₈ hydrochloride hydrate were purchased from Sigma-Aldrich, whereas sulfamethoxazole-d₄, azythromycin-d₃ and venlafaxine-d₆ from Toronto Research Chemicals (Ontario, Canada). Standard solutions were prepared on a weight basis in methanol (at a concentration of 1000 mg/L) and stored at - 20 °C. Ultra-pure water, acetonitrile and methanol LiChrosolv grade were from Merck (Darmstad, Germany). Working standard solutions containing all antibiotics were prepared in methanol/water (10:90, v/v) as well as isotopically labelled internal standards mixtures used for internal standard calibration.

2.3. Experimental set up

Five different experimental conditions were established in triplicate for each strain to evaluate the growth and removal of PhACs over 14 days (Table 1). Small microalgae aliquots from grown cultures were introduced into Erlenmeyer flasks containing 100 mL of GM. When the biomass live culture (A) was in the exponential growth phase (3-4 days), 0.1 mg/L of each PhAC was spiked in the A, C, D and E cultures, thereby obtaining a mixture of PhACs in each culture. Unitary samples for PhAC removal determination were taken just after spiking and homogenization, 7 days later and at the end of the experiment (14 days).

Table 1 shows the experimental conditions for algae cultures. Biomass live cultures (A and B) contained algae grown under light and dark conditions, respectively (24 h). Abiotic controls (C and D) contained sterilized medium without algae and were treated with continuous light irradiation or darkness; they helped to determine whether light, evaporation, photodegradation and other physicochemical processes had any effect on PhAC removal. A killed control (E) containing thermally inactivated algae (20 minutes at 121 °C) was irradiated under 24 h of to assess the contribution of sorption to the overall removal of pollutants.

Table 1. Experimental conditions and their respective abbreviations.

	Experimental condition	Culture	PhACs	Light
A	Biomass Light	Algae	+	+
B	Biomass Dark	Algae	+	-
C	Abiotic Light	-	+	+
D	Abiotic Dark	-	+	-
E	Killed Control	Killed algae	+	+

+ indicates the presence of PhACs and/or light; - indicates the absence of Algae, PhACs and/or light

2.4. Algal biomass concentration

Algae growth was monitored during the experiments A and B by measuring the optical density of the algal culture at 683 nm using a UNICAM 8625 UV/VIS spectrophotometer and converting the optical densities into algae dry weight for each sample. Correlations between weights and optical densities were previously obtained for each strain in previous experiments: TSS *C.reinhardtii* = $497.39 \cdot OD - 18.47$, $r^2 = 0.9862$; TSS *C.sorokiniana* = $192.41 \cdot OD + 0.34$, $r^2 = 0.9862$; TSS *D.tertiolecta* = $271.8 \cdot OD + 5.08$, $r^2 = 0.9883$; TSS *P.subcapitata* = $351.74 \cdot OD - 0.5553$, $r^2 = 0.9973$.

2.5. Determination of target compounds

Antibiotic compounds and venlafaxine were measured along 14 days by using an on-line turbulent flow liquid chromatography methodology coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, San Jose, CA), equipped with a Turbo Ion Spray source (ESI) in positive ionization mode. Samples were passed through a TFC Cyclone (50×0.5 mm, 60 μ m particle size; Thermo Fisher Scientific, Franklin, MA) so that high molecular weight compounds were flushed to the waste, whereas low molecular weight compounds got retained into the pores of the column particles. Chromatographic separation was performed in a Kinetex Biphenyl (50×2.1 mm, 2.6 μ m particle size; Phenomenex, Torrance, CA). More detailed information can be found in Sections S1 (SM).

2.6. Detection of transformation products

To ensure that degradation took place in the experiments, selected TPs were detected in water samples from the experiments performed with 2 microalgae (*Chlamydomonas reinhardtii* and *Chlorella sorokiniana*) and 3 antibiotics (azithromycin, sulfapyridine and erythromycin). Water samples were analysed using online turbulent flow chromatography coupled to an LTQ-Orbitrap hybrid high-resolution mass spectrometer (Thermo Fisher Scientific Company; Villebon-France). Extended information on sample preparation, instrumental methodology and TP occurrence can be found in Jaén-Gil et al. [43]. Suspect TPs were chosen based on results collected from literature sources.

3. Results and discussion

3.1. Growth results

Four microalgae strains were selected: *C. reinhardtii* and *P. subcapitata* because they are considered model algae for WW treatment [22]; *C. sorokiniana*, because the *Chlorella* genus is widely used, shows rapid growth and is highly efficient for WW nutrient removal [44]; and finally, *D. tertiolecta* because it grows in a marine environment instead of freshwater and has a high potential for biodiesel production [45]. Time-course profiles of cell growth for all 4 microalgae strains under light and dark conditions (A and B) are shown in Fig. 1. All the algae in the live conditions exhibited exponential growth, although each strain showed a different pattern, and as expected, the growth was affected by light. To evaluate the effects of the PhACs on algal growth, they were spiked in biomass light culture (A) at the time of the exponential growth phase, close to the maximum growth rate. After that moment, variations in algal growth were observed. The *C. reinhardtii* exponential growth phase started at 110 mg/L TSS, achieving a final value

of 703 mg/L (4 days after spiking with the contaminants). The cell concentration decreased after it reached a maximum on day 7, and the decrease continued until the end of the experiment on day 17. The algal growth profile under light irradiation differed from the profile previously reported by Hom-Diaz et al. [29] but was similar to that conducted by Zhou et al. [46], since light irradiation has a direct impact on growth in cell concentration.

The exponential growth phase of *C. sorokiniana* was similar to that of the *C. reinhardtii* culture. The exponential phase ended with a TSS concentration of 673 mg/L, and then the concentration exhibited a slight decrease and then maintenance at approximately 540 mg/L until the end of the experiment. There was no growth detected under dark conditions, and TSS was constant with a slight decrease during the following 14 experimental days.

D. tertiolecta growth did not have an exponential phase, but there was no visible lag phase, which may be because most of the cells were in the growth phase when they were transferred to the culture media. The growth rate increased until the end of the experiment, a profile similar to that published by Sánchez-Saavedra et al. [47]. Under dark conditions, low biomass growth was observed since no light was provided.

The *P. subcapitata* growth rate was lower than those of the rest of the strains under light conditions when the PhACs were spiked. The maximum TSS obtained was 189 mg/L. Similar results were obtained previously for this strain with the same GM but without PhACs [22], indicating that the low growth was not due to PhAC spiking.

Chen et al. [48] pointed out the importance of carrying out these studies when the objective is to select a pure strain to carry out PhAC degradation. They examined how fluoroquinolones and sulfonamides affected the growth of the green algae *Chlorella vulgaris* and the cyanobacterium *Chrysosporum ovalisporum*. Their results showed that

sulfonamides had no significant inhibitory effect on the growth of *C. ovalisporum* but had an inhibitory effect on *C. vulgaris*, whereas fluoroquinolones significantly inhibited *C. ovalisporum*.

Algae-based treatments of PhAC-containing WW show that a wide range of PhACs are not toxic to microalgae; however, not all microalgae can degrade all types of PhACs from WW. The selection of the microalgal strain is an important step in WW treatment and depends upon the type of PhACs present [23].

From these results, it can be concluded that these PhAC mixtures do not inhibit the growth of the selected algae strains.

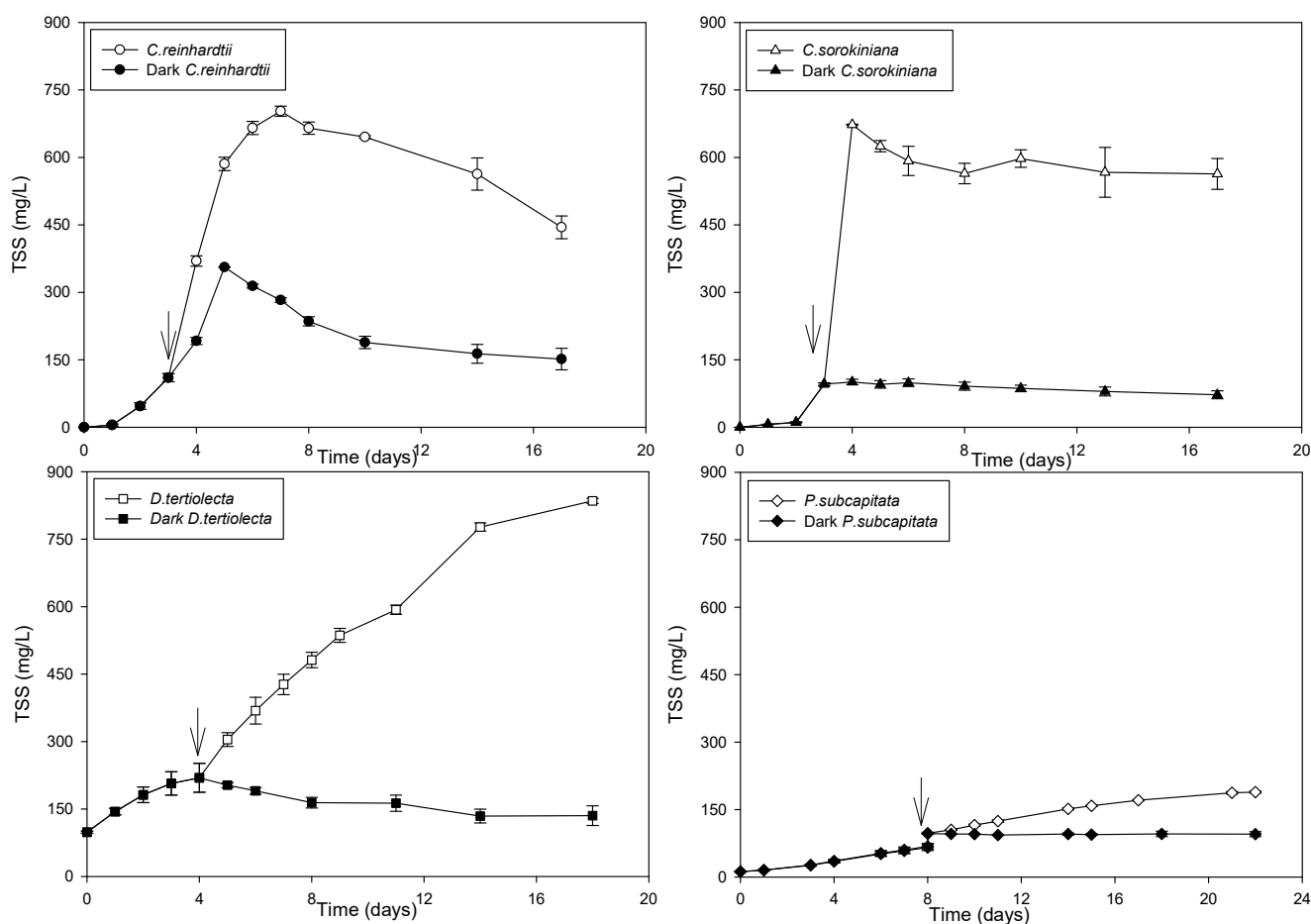


Figure 1. Time course growing profiles. (↓) indicates the moment when PhACs were spiked into the biomass light culture (condition A). Mean values of three replicates with the corresponding SD are shown.

3.2. *PhACs quantification*

A novel online TFC-LC-(ESI)-QQ-MS/MS methodology with automatic sample clean-up was developed for rapid quantification of the selected PhACs in microalgae water experiments. The optimum methodology was validated according to Commission Decision 2002/657/EC [49] for the three synthetic microalgae media used. The method validation parameters are presented in Section S2 (SM). The method limits of detection (MDL) for selected reaction monitoring (Section S1) and calculated by the lowest concentrate standard reached values up to MDL of 0.5 ng/L and MLQ of 1.65 ng/L (Table S3, Section S2). Method precision was determined from the repeatability and reproducibility percentages of relative standard deviation (%RSDs), with values below 20% in all cases (Table S4, Section S2). Additionally, the percentages of recovery with the online clean-up method were tested and ranged between 77% and 123% for TAP medium, 49% to 127% for artificial sea water and 74% to 112% for Bold 3N medium (Table S5, Section S2). This methodology was applicable for the study of selected antibiotics in microalgae water samples when a precleaning step was needed. Sample treatment and analysis were completed within a total run time of 6 minutes to prevent sample manipulation and contamination (Section S1).

3.3. Antibiotics removal

The use of four microalgae strains for removal of contaminants led to different removal efficiencies depending on the compound and the algae considered (Figures SF1-9, Section S3). In addition, the contribution of each of the removal mechanisms considered (abiotic transformations, biodegradation and sorption) was also different and depended on the algae and compound. Removal mechanisms were determined from the experiments conducted (Table 1). The contribution of photodegradation (Table 2) to removal was obtained by subtracting the dark abiotic removal percentage from that of light abiotic removal (eq. 1) since light abiotic removal includes not only photodegradation but also other nonspecific decomposition processes that also occur under dark abiotic conditions. This photodegradation mechanism is called direct photolysis, while during indirect photolysis, free radicals generated by algae during exposure to sunlight take part in contaminant degradation and only in live algae cultures with which indirect photolysis can take place [23].

The contribution of sorption for each alga (Table 3) was obtained by subtracting the abiotic light removal percentage from the killed control removal percentage (eq. 2) to account for the amount of pollutant sorbed onto algal cells without considering other abiotic processes (photodegradation and other abiotic removal processes).

$$\text{Photodegradation} = \text{Abiotic light} - \text{Abiotic dark} \quad \text{eq. (1)}$$

$$\text{Sorption} = \text{Killed control} - \text{Abiotic light} \quad \text{eq. (2)}$$

Nevertheless, it is worth mentioning that microalgae biomass changed during the thermal process; the colour changed (from green to yellow-brown), and as more days passed during the treatment, the suspension became clearer and allowed more light irradiance,

indicating that photodegradation as well as other decomposition processes (quantified in dark abiotic experiments) may occur together with sorption on algal biomass.

Biodegradation (Table 3) is not an isolated process; it occurs when the algae are alive, so the overall removal percentage under light conditions (A) includes those of biodegradation, photodegradation, sorption and other abiotic processes for each alga. Therefore, biodegradation, which includes indirect photolysis, is defined as the difference between the total elimination obtained under experimental conditions with light and live biomass (A) and the elimination obtained under killed control conditions with light (E), in which the phenomena of adsorption on dead biomass, photodegradation due to light and other abiotic phenomena such as volatilization occur, as shown in eq. 3.

$$\text{Biodegradation} = \text{Biomass Light} - \text{Killed control} \quad \text{eq. (3)}$$

Macrolides

Elimination of macrolides in the experiments with algae exposed to light (condition A) was very different depending on the algae considered: AZI was subject to higher removal percentages (between 30% and 67%) than CTM and ERY (lower than 37%) in all algae except for the *P. subcapitata* culture, with which the AZI removal percentage was 10%, a percentage similar to those for the photodegradations of the 3 macrolides AZI, CTM and ERY (Table 2). Macrolides were even eliminated in dark conditions (abiotic dark experiments) with elimination efficiencies ranging between 22% and 29%, which illustrates their low stability in aqueous solution. The contributions of sorption were higher; between 20% and 37% sorption percentages were calculated for AZI in the *C. sorokiniana* and *C. reinhardtii* experiments, respectively (Table 3). These results are in accordance with those obtained by Kobayashy et al. [50], who concluded that sorption onto activated sludge was the main mechanism for removal of AZI in WWTPs.

The ERY removal efficiency obtained with all algae in the biomass experiments run with light exposure (A) was approximately 30% (Table 3). On the other hand, sorption was negligible for all strains, with biodegradation levels between 1 and 23%.

The role of biodegradation in the elimination of macrolides by the algae *C. reindhardtii* and *C. sorokiana* was confirmed by the presence of TPs from AZI and ERY (Table S6 in Section S4), which are the antibiotics that exhibited the highest removal in algal light experiments. For instance, TP734 and TP733 were only detected in the two irradiated live algae cultures (condition A) and were reported to be mainly formed through algal biodegradation mechanisms involving AZI and ERY, respectively [43]. Moreover, other studies have also reported that these TPs are associated with biodegradation processes [42–45]. Other TPs of AZI, such as TP590 and TP433, were also reported to be generated because of the abovementioned instability of macrolides in aqueous solution, and they were actually found in abiotic conditions (C and D) [43]. Both compounds were also described in the experiments of Tong et al. [54] and attributed to the photodegradation of AZI. Likewise, the ERY TPs TP749 and TP747 were only found in the abiotic experiments but not in the light algae cultures (A), in contrast with the findings of Llorca et al. [41], who identified them as ERY degradation products formed after enzymatic treatment by EreB esterase.

Table 2. PhACs percentage removal contribution (%) of photodegradation and other abiotic mechanisms (abiotic dark conditions). Mean values of three replicates with the corresponding SD are shown.

Compound	Abiotic conditions		
	Abiotic Light	Abiotic Dark	Photo-degradation
AZI (macrolide)	23±12	22±16	1±20
CTM (macrolide)	21±33	22±32	0
ERY (macrolide)	34±14	29±14	5±20
CFC (fluoroquinolone)	97±2	0±6	97±6
OFC (fluoroquinolone)	85±18	7±7	78±19
NFC (fluoroquinolone)	95±7	8±6	87±9
TMP	14±15	0±9	14±17
SPY	34±7	1±6	33±17
PMA (pyridopyrimidine)	87±2	7±13	78±13
VFX (antidepressant)	4±5	6±9	0

Table 3. PhACs removal percentages (%) and removal contribution (%) of sorption and biodegradation mechanisms after 14 days of treatment under different experimental conditions. (“–” indicates negative values). Mean values of three replicates with the corresponding SD are shown.

	<i>C. reinhardtii</i>				<i>C. sorokiniana</i>				<i>D. tertiolecta</i>				<i>P. subcapitata</i>			
Compound	Total removal	Killed control	Sorption	Bio-degradation	Total removal	Killed control	Sorption	Bio-degradation	Total removal	Killed control	Sorption	Bio-degradation	Total removal	Killed control	Sorption	Bio-degradation
AZI (macrolide)	67±11	60±8	37±14	-	58±11	43±4	20±13	15±12	30±2	19±16	-	11±16	10±10	0	-	10±10
CTM (macrolide)	36±5	38±8	17±34	-	0±9	0	-	0±9	18±8	32±5	11±33	-	20±3	0	-	20±3
ERY (macrolide)	32±2	20±7	-	12±7	33±5	10±18	-	23±19	30±16	29±11	-	1±19	30±7	23±4	-	7±8
CFC (fluoroquinolone)	76±8	76±4	-	0±9	51±5	90±4	-	-	100±0	100±0	3±2	0	77±4	93±3	-	-
OFC (fluoroquinolone)	32±16	75±8	-	-	22±7	80±3	-	-	88±2	98±1	13±18	-	53±3	39±3	-	14±4
NFC (fluoroquinolone)	73±5	77±3	-	-	46±4	83±4	-	-	100±0	100±0	5±7	0	65±1	77±2	-	-
TMP	20±16	62±7	48±17	-	11±8	17±12	3±19	-	34±2	25±11	11±19	9±11	26±3	24±5	10±16	2±6
SPY	93±2	84±2	50±7	9±3	73±3	73±4	39±8	0±5	56±5	35±24	1±25	21±25	48±3	50±1	16±7	-
PMA (pyridopyrimidine)	71±3	66±13	-	5±13	57±10	79±1	-	-	85±3	83±1	-	2±3	77±4	64±6	-	13±7
VFX (antidepressant)	17±7	0	-	17±7	6±2	0	-	6±2	4±2	0	-	4±2	11±5	1±8	-	10±9

Fluoroquinolones

For the three fluoroquinolone antibiotics studied (CFC, OFC and NFC), *D. tertiolecta* led to the highest removal percentages (>88%), whereas *C. sorokiniana* had the worst fluoroquinolone removal percentages of <51% (Table 3). Photodegradation in the case of fluoroquinolones exhibited removal values higher than 78% (Table 2), whereas abiotic losses under dark conditions accounted for less than 8% removal. These results show how photosensitive these compounds are, and this has been previously noted [55]. Babic et al. [48, 49] also proved how quickly CFC and NFC were photodegraded in experiments with different matrices. Zhang et al. [50, 51] studied the photodegradation of NFC in aqueous solution containing the microalgae strain *C. vulgaris*. The presence of this algae enhanced NFC removal. They suggested that algae generate lower molecular weight organics that produce hydroxyl radicals under UV irradiation and support enzymatic reactions occurring on the algae surface, which favour NCF removal. In the same study, a higher algae concentration led to higher NFC removal in a shorter period of time, as reported by Dorival-García et al. [58] on quinolone removal from sludge. In killed control cultures (condition E), high removal percentages were obtained for most of the strains, but as stated above, the removal in the killed control was due mainly to sorption and photodegradation phenomena. In this case, considering the contribution of photodegradation (Table 2), it can be concluded that sorption is not the most important mechanism for removal in the presence of light. This issue was previously reported by Hom-Díaz [59], who confirmed in several kinds of experiments that CPX removal was significantly enhanced by irradiation.

Other antibiotics

Trimethoprim (TMP) removal under biomass light conditions (A) was quite moderate (< 34%) (Table 3), with all the algae tested, with photodegradation contributing less than

15% (Table 2). Sorption percentages (Table 3) were exhibited by the different algal strains; while 48% was sorbed onto *C. reinhardtii*, and only 3% was sorbed onto the *C. sorokiniana* cell wall, which indicates that the different compositions of the algal cell wall have a great impact on the absorbance of TMP.

In several studies, it was found that TMP is quite resistant to biological treatment of wastewater [62, 63], while other studies reported partial removals of TMP ranging from 40 to 50% [62–64] and even nearly complete removal [18]. Even with differences in removal efficiencies, biodegradation has been observed and is often attributed to removal under aerobic (nitrifying) conditions [64, 67]. In the present study, low levels of TMP biodegradation were observed (Table 3) by other authors. Yu et al. [66] observed low biodegradability; 27% of TMP was removed via biodegradation, and 40% was removed from the aqueous phase by biosorption or sorption during a 14-day experiment with an immobilized cell process, whereas volatilization and hydrolysis were negligible. Jewell et al. [67] studied the variability of TMP removal in an activated sludge system by treating municipal wastewater and found that formation of transformation products was influenced by spiking concentrations. Some of the TPs were formed due to hydroxylation and several subsequent redox reactions, some were formed by oxidation and some TPs were identified as photodegradation products of TMP. However, some authors pointed out that, apart from biotransformation, photolysis represents a possible mechanism [64,68], whereas others stated the need for TMP oxidation prior to biological treatment [69]. Lower removal percentages, ranging from 20 to 30%, have been detected in biofilm systems [70,71]. Therefore, from the previous results, it can be concluded that TMP is a very stable compound exhibiting low biodegradation and photodegradation percentages. In our study, biodegradation efficiency was lower than 10% (Table 3), and photodegradation efficiency was lower than 15% (Table 2).

In the present experiments with algal treatment (A), complete removal of SPY was almost achieved in *C. reinhardtii* culture (93%), whereas lower removal levels were observed with *C. sorokiniana* (73%), *D. tertiolecta* (56%) and *P. subcapitata* (48%) cultures (Table 3). Abiotic degradation caused 34% elimination, as observed under abiotic light conditions (Table 2); this was mainly attributed to photodegradation. Sorption and biodegradation contributed to total removal in a different way for the different algae; sorption contributed more than biodegradation in removal of *C. reinhardtii*, *C. sorokiniana* and *P. subcapitata*, while for *D. tertiolecta* sorption was negligible, with a biodegradation contribution of approximately 21% (Table 3).

The presence of TPs in the experiments resulting from SPY biodegradation and/or photodegradation processes was confirmed: two TPs from SPY were detected in experiments performed with *C. reinhardtii* and *C. sorokiniana* (Table S6, Section S4). TP185 was detected in all experiments except those carried out in dark conditions (abiotic dark). The generation of this TP can thus be linked to photodegradation processes [43], although the contribution of biodegradation cannot be discarded. TP185 was detected as the main TP generated from SPY during experiments using UV/H₂O₂ treatment [72] and was detected after 48 h of treatment of contaminated water with the white-rot fungus *Trametes versicolor*, which also resulted in complete removal of SPY [73]. In contrast, TP265 was only detected in live light experiments and thus was correlated with biodegradation processes. However, TP265 was also detected after treatment of water contaminated with SPY with the white-rot fungus *Trametes versicolor* [73].

High removal percentages were obtained for pipemidic acid (PMA) during irradiation of algal solutions (A): these were 85% for *D. tertiolecta* culture, 77% for *P. subcapitata*, and 71% for *C. reinhardtii*, whereas *C. sorokiniana* only effected 57% removal (Table 3). The photodegradation contribution was very high in the case of PMA (78%), with a

percentage similar to those for the removal killed controls (between 64 and 83%) with all algae strains. Abiotic degradation caused 87% elimination, as observed under abiotic light conditions, with 78% attributed to photodegradation and 7% attributed to other abiotic phenomena (Table 2). Based on these results, sorption and biodegradation did not appear to be prevalent elimination mechanisms.

The use of pure microalgae strains for antibiotic removal can be effective since removal of such compounds was achieved, even though most of the antibiotics tested are photosensitive and photodegradation is the major mechanism occurring in the system. This is in accordance with other authors who stated that direct and indirect photolysis appears to be a major mechanism for removal in algae treatment systems [35, 66]. Although microalgae may not contribute to all PhAC biodegradation, treatment in algal ponds and photobioreactors, where illumination is required for biomass growth, could enhance the removal of light-sensitive micropollutants. Moreover, the microalgal biomass produced could be further used for biofuel production [64].

Venlafaxine

Finally, the antidepressant venlafaxine was tested, and its persistence under biological treatment was confirmed using pure algae strains, which exhibited removal percentages lower than 17% (Table 3 and Figure SF10, Section S3). On the other hand, the overall removal efficiency can be attributed to biodegradation or other abiotic processes rather than photodegradation, since sorption and photodegradation contributions were negligible (Tables 2 and 3). The persistence of this compound in different WWTPs has been previously reported by other authors, with removals ranging between 18 and 40% [74–76]; the use of other biological technologies such fungal treatment [77] exhibited removal efficiencies between 55 and 70% in batch studies with venlafaxine spiked at 50 µg/L and 5 mg/L, respectively [78].

It is crucial for biorefineries to valorise the residual algal biomass used in wastewater treatment to guarantee the economic feasibility of the processes [79,80]. In this sense, anaerobic digestion is among the most straightforward and suitable technique for energy recovery [81]. Depending on the algae strain, the biomass will take up more or less contaminant, and algae with a higher degradation capacity and low adsorption capacity should be chosen for such processes. The effect of nondegraded PhACs taken up by microalgae biomass on methane production by anaerobic digestion is being studied. Similar studies have been carried out by the authors with nonpolar pesticides. Algae biomass contaminated with pesticides was employed as feedstock for biofuel production through anaerobic digestion, resulting in biogas yields similar to those of fresh algae biomass [82].

4. Conclusions

The current results showed that microalgae growth depends on the algae strain, and none of the algae was inhibited by the presence of PhACs. In general, good removal percentages were obtained for all the antibiotics with the algae tested, with lower removals for TMP (between 11 and 34%) in all cultures and higher removals for fluoroquinolones in *D. tertiolecta* cultures (between 88 and 100%). Macrolides were mainly removed by and biodegradation rather than photodegradation (which contributed less than 5%). The highest biodegradation removals were observed for ERY (23%), SPY (21%) and CTM (20%). For fluoroquinolones, and PMA the main removal mechanism was photodegradation (>78%). TMP is a very stable compound exhibiting low biodegradation (< 10%) and photodegradation (< 15%) percentages; this was also true for the antidepressant VFX. The presence of TPs for the macrolide AZI and SPY in algal experiments only and not in abiotic experiments confirmed that biodegradation of these

parent compounds occurred in those experiments. Although photodegradation greatly contributed to antibiotic removal in microalgae-based systems, biodegradation processes also took place. These systems present a double benefit, with biodegradation of pollutants and the production of biomass for further uses.

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CRedit Author Contributions

Andrea Hom-Díaz: Investigation, visualization, formal analysis, writing: original draft and writing: review and editing. Paqui Blázquez and Teresa Vicent: Funding acquisition, methodology, supervision, conceptualization, and writing: review and editing. Adrián Jaén: Investigation, formal analysis, writing: original draft and writing: review and editing. Sara Rodríguez-Moraz: Funding acquisition, and writing: review and editing. Damià Barceló: supervision and conceptualization.

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