Biodegradation of hydrophobic pesticides by microalgae: transformation products and impact on algae biochemical methane potential

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Abstract

Intensive and extensive use of pesticides has contributed to their wide distribution in soil, air, and water. Due to their detrimental effects on non-target organisms, different technologies have been considered for their removal. In this work, three hydrophobic pesticide active compounds, namely, chlorpyrifos, cypermethrin, and oxadiazon, were selected to study the potential for their removal from aqueous media by a microalgae consortium. An abiotic and a killed control (thermally inactivated dead microalgae biomass) were employed to clarify their removal pathways, and pesticide content was quantified in liquid and biomass phases for 7 days. At the final time, total degradation (biodegradation plus photodegradation) contributed to the removal of 55% of oxadiazon, 35% of chlorpyrifos, and 14% of cypermethrin. Furthermore, more than 60% of chlorpyrifos and cypermethrin were removed by sorption onto microalgae biomass. Overall, the three pesticides showed high removal from the liquid phase. O,O-diethyl thiophosphate was identified in the liquid phase as a transformation product of chlorpyrifos formed by microalgae

degradation. Phycoremediation was coupled with anaerobic degradation of the microalgae biomass containing the retained pesticides by sorption through biochemical methane potential tests. Anaerobic digestion was not inhibited by the pesticides as verified by methane production yields. The removal efficiency of the pesticides in the digestate was as follows: chlorpyrifos > cypermethrin > oxadiazon. These results highlight the potential of low-cost algal-based systems for the treatment of wastewater or effluents from agrochemical industries. The integration of wastewater treatment with biogas production through anaerobic digestion is a biorefinery approach that facilitates the economic feasibility of the process.

Keywords

Biodegradation - Phycoremediation – Microalgae – Agrochemicals – Metabolites – Biochemical methane potential

1. Introduction

Concerns about the adverse effects of agrochemicals in the environment are public and widely known. The broad use of pesticides in agriculture leads to diffuse contamination through spray drift and runoff, which contributes to their distribution in soil, air, and water. Likewise, point sources of pesticides include their use in public health, green areas, livestock and other industries, and households. Once pesticides are released into the environment, their fate, mobility, and transformation are influenced by complex physical, chemical, and/or biological processes, such as degradation, volatilisation, accumulation in soil, uptake by plants or microorganisms, and transport to ground and surface waters [1]. Moreover, their distributions in soil, water, and air are affected by transfer between phases and adsorption/desorption processes [2]. Pesticides taken up by living organisms are susceptible to bioaccumulation [3], co-metabolic or partial transformation into other degradation compounds, or mineralisation [4,5].

The European Drinking Water Directive 98/83/EC defined 0.1 µg L^{-1} as the threshold for a single pesticide and 0.5 µg L^{-1} as the threshold for total pesticides in human water consumption. Conventional processes in wastewater treatment plants (WWTPs) are not efficient in the

degradation of exogenous pollutants because they are not designed for these purposes [6,7]. Specially hydrophobic compounds are removed through sorption and accumulation in waste activated sludge (WAS) [8], which could later be involved in other processes such as anaerobic digestion or composting. However, if WAS is not previously treated and xenobiotics are not degraded, they can accumulate in the media. Other conventional physical and chemical removal treatments such as nanofiltration, advanced oxidation processes, and adsorption on activated carbon are effective but expensive [9,10]. Over the past few years, assessments of the potential of microalgae for directly transforming or enhancing the biodegradation of emerging contaminants, heavy metals, and pesticides from wastewater is gaining attention among researchers [11–13]. Microalgae-based wastewater treatment systems can facilitate algae uptake, algae-mediated photolysis, bioaccumulation, and intracellular and extracellular biodegradation of pollutants [14–16]. Microalgae can degrade complex parent compounds to simpler molecules, highlighting their substantial biodegradation potential [17]. Moreover, phycoremediation is a low cost and solar power-driven process that can be coupled with nutrient removal and bioproduct recovery [12,18]. In algae-based systems, such as open ponds and closed photobioreactors, microalgae produce the O_2 required by heterotrophic aerobic bacteria using CO_2 released by these microorganisms [19]. Synergetic interactions between microalgae, bacteria, and diverse microorganisms enhance the detoxifying potential of these systems [17]. Therefore, microalgae-mediated bioremediation systems do not require the addition of carbon sources or nutrients in stoichiometric balance, as required by bacteria and fungi [17].

Among chemical families of pesticides, organophosphates and oxadiazol are widely employed substances with effects on non-target organisms. Chlorpyrifos (CHL) is a chlorinated organophosphate insecticide for pest control that is used broadly for a great variety of crops [20,21]. Despite having a moderate persistence, these pesticides are highly toxic to mammals, aquatic invertebrates, freshwater fish [5,22], and pollinisers, and they have neurotoxic, immunological, and psychological effects in humans [4]. In January 2020, the European Commission did not renew its approval of CHL (Regulation (EU) 2020/18). Cypermethrin (CYP) is a pyrethroid insecticide used in pest control worldwide that is approved in the European Union. Recently, the toxic effect of CYP on pollinisers [23] and the negative effects on the fertility, immune system, cardiovascular system, and hepatic metabolism of mammals [24] were

confirmed. Oxadiazon (OXA) is a selective pre-emergence oxadiazoline herbicide against annual dicotyledons, which is also used as a post-emergence measure against broad-leaved weeds [25,26]. Due to its potential risks, OXA was included in the Watch List of Substances established by EU Decision 2015/495, but this compound was removed from the second list (2018/840/EU). Despite OXA being toxic to aquatic microorganisms [27], it continues to be detected in bodies of water worldwide [28]. In humans, OXA could be associated with liver cancer and harmful effects on reproductive and endocrine functions [29].

Some authors have highlighted the potential of a microalgae–bacteria consortium for the removal of xenobiotics, and some have studied degradation of pesticides using microalgae under sterile conditions [30,31]; however, maintaining these conditions when scaling-up could be difficult and the interaction effects between microorganisms and xenobiotic compounds have not been evaluated. This work focused on assessing the potential of a microalgae consortium to degrade three hydrophobic pesticides, CHL, CYP, and OXA, studying the evolution of their concentrations in the liquid and solid phases while identifying their transformation products (TPs). With the aim of biomass valorisation, the effect of the non-degraded pesticide taken up by the microalgae biomass in methane production by anaerobic digestion was studied.

2. Materials and methods

2.1. Chemicals and reagents

The pesticides studied include three hydrophobic (log K_{ow} > 4) compounds: CHL [(O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate], CYP [α-cyano-3-phenoxybenzyl ester of 2, 2-dimethyl-3-(2, 2-dichlorovinyl) 2-2- dimethyl cyclopropane carboxylate], and OXA [5-terbutyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one] (properties are provided in Table 1). Stock solutions of each pesticide in methanol were prepared using analytical standards purchased from Sigma-Aldrich (Steinheim, Germany) and stored in the dark at -20 °C until use. Internal standards of d_{10}-chlorpyrifos and phenoxy-d_5-fenvalerate were also purchased from Sigma-Aldrich; d_7-oxadiazon was purchased from LGC standards (Teddington, Middlesex, UK). Ethyl acetate, acetone, water, dichloromethane, hexane, and methanol solvents were purchased form J.T. Baker (Waltham, Massachusetts, USA). Chloroform was purchased from Carlo Erba (Val De Reuil, Eure, France). Formic acid (98–100%) was purchased from Merck (Darmstadt,

Germany). The quality of all solvents was according to organic trace analysis. Sulphuric acid was obtained from Scharlau (Spain), and mineral salts and other chemicals were purchased from Scharlab (Spain). GF/A and CG/C glass fibre filters were obtained from Whatman (GE Healthcare, USA).

Table 1
[a] [32], [b] [33], [c] [34], [d] [35], [e] [36]

2.2. Microalgae cultures

The microalgae samples employed in the experiments were collected from a 1 m^3 outdoor tubular semi-open photobioreactor (PBR), described previously by Hom-Diaz et al. [37], operating in semi-continuous mode with feeding once per week using Bold Basal Medium. The microalgae employed in the degradation experiments were taken 24 h after feeding. The consortium was composed mainly of *Chlorella* sp. and *Scenedesmus* sp., morphologically examined using an optical microscope (Zeiss, AixoCam ERc 5s), although other microorganisms such as bacteria and protozoa were present, as reported previously for outdoor microalgae-based systems [37–39].

Biomass evolution along experimental time was determined by optical density (OD) and was correlated to total suspended solids (TSS) concentration according to Eq. 1:

$$TSS\ (g\ L^{-1}) = 0.7565 \times OD_{680} - 0.0422 \quad (r^2 = 0.962) \qquad (1)$$

2.3. Pesticides removal by microalgae consortium

Batch biodegradation experiments of the target pesticides were performed in 250 mL Erlenmeyer flasks containing 100 mL of microalgae solution from the PBR described previously. Flasks were under orbital agitation (100 rpm) in a 25 ± 1 °C temperature-controlled chamber. A standard solution of the target pesticide in methanol was spiked to the reactors to a final concentration of 1 mg L^{-1}. In addition to the flask containing the microalgae consortium and the studied pesticide (microalgae reactor), a killed control (dead microalgae biomass) employing thermally inactivated biomass (autoclaved at 121 °C for 20 min) was used to determine the removal by sorption onto biomass. The influence of other losses was assessed by an abiotic control containing the

pesticide in absence of microalgae. A blank reactor with the microalgae consortium without the pollutants was used to assess the chemical effect on biomass growth. OD was monitored in the microalgae reactors as an indicator of microalgae biomass growth. TSS and volatile suspended solids (VSS) were determined from the blank reactor at the beginning and the end of the experiments. All conditions were assayed under the influence of light during a 24 h photoperiod (31 µmol photon m^{-2} s^{-1}) provided by cool white fluorescent lamps. Bearing in mind that the studied xenobiotics are non-polar molecules with a great partition from the aqueous phase into organic solvents, determination of pesticide concentration was carried out at 2 and 7 days of exposure in the aqueous and the solid phase. Identification of the TPs was performed in the liquid phase at 0, 2, and 7 days. The study conditions were evaluated independently for each pesticide in triplicate. At each sampling time, a triplicate of each condition was used to perform analytics, while the other reactors remained until the next sampling time. Biodegradation experiments were not performed in aseptic conditions.

The removal kinetics of the pesticides was adjusted to a first-order reaction model [31], according to Eq. 2:

$$C = C_0 * \exp(-k * t) \quad (2)$$

where C_0 and C are the initial and final (t = 7 d) concentrations of the pesticide in the solution, respectively, k is the kinetic rate constant (d^{-1}), and t is the removal time (d). The theoretical half-life ($t_{1/2}$) of pesticides was calculated as ln2/k (d) [31].

2.4. Biochemical Methane Potential (BMP) tests

The potential methane production was evaluated by biochemical methane potential (BMP) tests employing a concentrated microalgae suspension from the biodegradation experiments at the final time. A volume of 500 mL of solution from the microalgae flasks was sedimented naturally for 12 h. Later, the supernatant was removed, and 250 mL of the settled biomass was employed as a substrate for the BMP tests.

Anaerobic batch assays were performed according to a previously described procedure [40], taking into account suggestions from other authors [41,42]. BMP tests were performed

considering an inoculum-to-substrate ratio (ISR) of 2 based on VS [40]. Inoculum (total solids (TS) = 16.6 ± 0.4 g L^{-1} and volatile solids (VS) = 10.2 ± 0.2 g L^{-1}) was collected from the anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona) and pre-incubated at 37 °C for 13 days to reduce background production of biogas. The assays were carried out at mesophilic conditions (37 °C) in triplicate using 120 mL glass bottles. They were filled with inoculum, substrate, and tap water until the working volume (80 mL) was attained. Subsequently, reactors were flushed with pure N_2 to ensure anaerobic conditions, closed with a gastight butyl rubber septum, and incubated in a temperature-controlled chamber. Biogas production and accumulation in the headspace of the bottles were measured employing an SMC pressure switch manometer (1 bar, 5% accuracy) until biogas generation ceased. Blank reactors (containing only inoculum) were used to calculate the background biogas production of the inoculum. Net biogas production was determined by subtracting the biogas production of the blank reactor from the gross biogas production of microalgae reactors. Additionally, crystalline cellulose was used as a substrate in control reactors to assess the biological activity of the inoculum. Reactors were shaken manually before each pressure measurement. Periodically, the methane content of the generated biogas was analysed by gas chromatography. Results of the BMP tests were expressed as the volume of methane generated per mass of VS of the added substrate (mL CH_4 g VS^{-1}) under standard temperature conditions (273.15 K and 1.0133 bar).

The modified Gompertz equation [43] was employed to model the biomethane production and calculate kinetic parameters according to Eq. 3:

$$P_{net}(t) = P_{max}.\exp\left\{-\exp\left[\frac{R_{max}.e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (3)$$

where $P_{net}(t)$ is the net cumulative methane yield (NmL CH_4 g VS^{-1}) at time t, P_{max} is the methane yield potential (NmL CH_4 g VS^{-1}), R_{max} is the maximum daily methane production rate (NmL CH_4 g VS^{-1} d^{-1}), t is the digestion time, and λ represents the lag phase (d). The hydrolysis rate was estimated using a first-order kinetic model [44], as shown in Eq. 4:

$$B = B_0.[1 - \exp(-K_H.t)] \quad (4)$$

where B is the cumulative methane yield (mL CH_4 g SV^{-1}), B_0 is the ultimate methane yield (mL CH_4 g SV^{-1}), t is the digestion time (d), and K_H is the hydrolysis rate (d^{-1}). Kinetic analyses were performed using the software Matlab R2015a (MathWorks Inc., Natick, MA, USA).

2.5. Analytical methods

2.5.1. Quantification of pesticides and identification of TPs

At each sampling time (days 0, 2, and 7), the total volume was removed from three flasks. To determine the pesticide concentration in the liquid phase, a fibreglass filter (1.6 µm, GF/A, Whatman) was used to separate biomass from the aqueous phase and then 50 mL of the filtrate was spiked with internal standards (d_{10}-chlorpyrifos, phenoxy-d_5-fenvalerate, and d_7-oxadiazon) to a final concentration of 0.1 mg L^{-1}. The liquid samples were collected in amber glass tubes, while the biomass cake retained by the filter was collected with a spatula and stored inside aluminium bags to evaluate later the concentration of pesticide in the biomass phase. Additionally, to determine the pesticide sorption or retention during the filtration, the pesticide concentration in the employed filters was determined. All samples were frozen at -20 °C until analysis.

A 30 mL volume of liquid sample was ultrasonically extracted for 5 min with chloroform. Then, the sample was centrifuged (3.500 rpm, 5 min) and the organic phase was recovered. The aqueous phase was extracted once more and the organic phases were evaporated until dry under a nitrogen stream. The residue was reconstituted with 50 µL of ethyl acetate and then subjected to gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) analysis.

Freeze-dried biomass samples and filters were extracted by pressurised liquid extraction using a 350 ASE system (Dionex, USA). Prior to extraction, biomass samples were spiked with 15 ng of internal standard and left overnight at 4 °C. Then, samples and 2 g of Florisil were loaded into an ASE extraction cell (22 mL) previously filled with 6 g of Florisil. Hexane and dichloromethane (1:1, v/v) were used as the extraction solvent. Temperature and pressure were set at 100 °C and 1650 psi, respectively. Extracts were evaporated to dryness under a nitrogen stream, and the residue was reconstituted with 50 µL of ethyl acetate prior to GC-MS/MS analysis.

Pesticide concentrations were determined through GC-MS/MS on a 7890B GC coupled to a 7000C triple quadrupole (Agilent technologies, USA) equipped with a DB-5MS capillary column

(30 m × 0.25 mm, 0.25 µm). The operating conditions were 80 °C for 2 min, raised at 25 °C min^{-1} to 180 °C for 6 min, at 5 °C min^{-1} to 240 °C for 5 min, at 10 °C min^{-1} to 280 °C for 5 min, and at 30 °C min^{-1} to 325 for 2 min. The temperatures corresponding to the transfer line and the ionisation source were 300 and 280 °C, respectively. The collision energy was 70 eV. Two different transitions were monitored for each pesticide.

No analytes of interest were observed in the method blank samples. Recoveries ranged from 50 to 58%. Detection limits were 1.7, 2.8, and 0.9 ng L^{-1} for CHL, CYP, and OXA, respectively. Limits of quantification were 5.6, 9.2, and 2.1 ng L^{-1} for CHL, CYP, and OXA, respectively.

Identification of TPs was carried out on a Waters Acquity UHPLC system (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific; San Jose, CA, USA) equipped with a HESI II heated-electrospray ionisation source. Chromatographic separation was performed on a Purospher STAR RP-18 end-capped (2 µm) Hibar HR 150-2.1 UHPL column (Merck). The mass spectrometer performed a Fourier transform mass spectrometry scan event of 50-700 m/z at a resolution of 70,000 and a subsequent MS/MS scan event was acquired at a resolution of 35,000. To identify all potential TPs, the total ion current chromatograms acquired at 2 and 7 days were compared with those obtained at initial time using Compound Discoverer (Thermo Fisher Scientific). This software allows differential analysis of selected sets of samples by simultaneously comparing thousands of MS spectra to find significant differences between the control and samples. The accurate masses of the potential TPs were then extracted to confirm their presence. Identification of the potential TPs was based on their accurate mass, mass error, molecular formula, and degree of unsaturation of the parent ion and product ions.

2.5.2.Other analyses

Biogas composition (carbon dioxide and methane content) was analysed using a gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Mississauga, Canada) equipped with a thermal conductivity detector and a Supelco Porapack Q column (3 m x 3.2 mm) (Pennsylvania, USA). Helium was the carrier gas (338 kPa). Oven, injector, and detector

253 temperatures were 70, 150, and 180 °C, respectively. Samples were injected with a 100 µL
254 syringe (VICI PS Syringe A-2, 0.74 mm x 0.13 mm x 50.8 mm).

255 Acetic, propionic, and butyric acid concentrations were determined using a Dionex 3000 ultimate
256 high-performance liquid chromatography system (Barcelona, Spain) equipped with a UV/visible
257 detector (210 nm). The chromatographic separation was performed in an ICE-COREGEL 87H3
258 column (7.8 x 300 mm, Transgenomic, USA), heated at 40 °C, employing 0.006 mM of H_2SO_4 as
259 a mobile phase at a flow rate of 0.5 mL min^{-1}. Samples were previously centrifuged (10 min, 8000
260 rpm, Beckman Coulter, Avanti J20 XP) and then filtered by 0.45 µm nylon syringe filters.

261 TS, VS, TSS, and VSS were determined following the procedures described in the Standard
262 Methods [45]. pH was measured using a pH meter (Crison, Spain), and OD was determined by
263 spectrophotometer (DR3900, Hach).

264 ### 2.6. Data analysis

265 The experimental data were analysed statistically using a one-way analysis of variance (ANOVA)
266 of repeated measures, followed by a Bonferroni post-hoc test when significant differences were
267 identified ($p < 0.05$). P-values represent Bonferroni corrected significance levels. Statistical
268 calculations were carried out with R (version 3.6.3).

269 ## 3. Results and discussion

270 ### 3.1. Biodegradation of pesticides by microalgae consortium and TP identification

271 The characterisation of the initial microalgae biomass employed in batch experiments is shown
272 in Table 2. The biodegradation potential of the non-polar pesticides by the microalgae consortium
273 was evaluated by studying their behaviour under the following conditions: microalgae reactor,
274 killed control, and abiotic control.

275 Table 2

276

277 Figure 1 shows the evolution of the pesticide distribution in the liquid and solid phases in the
278 microalgae reactors. The remaining fraction of the target pesticide in the liquid and in the solid

279 phases through time was calculated as the relation between the residual quantity of the pesticide
280 in the liquid or solid phase and the total initial amount of pesticide in both phases. The solid phase
281 included the biomass contained in the flask and the filter used for biomass separation. Sorption
282 of the target pesticide onto the filter was determined for the microalgae reactors (1323.3 ± 131.3
283 ng of CHL, 11509.7 ± 3027.4 ng of CYP, and 920.3 ± 235.3 ng of OXA), and these values were
284 also applied to killed reactors. At the initial time, the three pesticides were mainly detected in the
285 liquid phase, where they were added; however, the residual pesticide distribution changed
286 gradually with time in the solid phase due to sorption onto the algae biomass. These compounds
287 have a tendency for sorption on solids and biosolids in concordance with their large log K_{ow} value
288 and low solubility in water. They present a high solubility in lipids and an affinity for the microalgae
289 cell wall [46]. For instance, sorption onto the solid phase was also observed for pyrimethanil (a
290 fungicide with high log K_{ow} value) when it was in contact with microalgae [47]. The pesticide
291 retained by the solid phase accounted for 62%, 60%, and 33% of the CHL, CYP, and OXA,
292 respectively (Fig. 1), highlighting the role of sorption in pesticide removal from the liquid phase.
293 The concentration of CHL in the aqueous media was reduced by 11 times after 2 days. The OXA
294 concentration in the solid phase remained fairly constant from day 2 to day 7 (36% to 33%). OXA
295 was gradually removed from the liquid phase due to sorption and degradation (by day 7, its
296 concentration in the liquid phase decreased by 8-fold).

297

298 Figure 1

299 (two-column fitting image)

300

301 Table 3 shows the distribution of the pesticides in the killed and abiotic controls after 7 days of
302 exposure. The remaining fraction of CHL in the solid phase of the killed control at the end of the
303 experiment (45.9 ± 11.5%) was lower than that in the microalgae reactor (61.9 ± 5.8%). With
304 regard to OXA, it showed a higher sorption capacity of the inactivated biomass (63.8 ± 32.1%) in
305 comparison with the active biomass (32.8 ± 12.7%). The cell wall of microalgae is constituted by
306 an aggregation of polymers with functional groups over its surface (such as carboxyl, phosphoryl,

and amine) that give it a negative charge [17]. Bearing in mind that the structure of the microalgae surface is modified after heat treatment [48] for inactivation, sorption on the active biomass (microalgae reactor) could be different from sorption on the inactivated biomass (killed control). Furthermore, the absence of an active enzyme system after the biomass has been thermally inactivated [14] could lead to dissimilarities when both conditions are compared. Additionally, some authors have reported a major contribution of dead algal cells in the photolysis of xenobiotics [14,49]. Luo et al. [14] proved that chlorophyll is the major active substance in dead cells and generates singlet oxygen that acts as a catalyst stimulating and accelerating the photodegradation of xenobiotics under light irradiation, which could also explain the removal differences in both conditions. Concerning CYP, sorption onto solid phase was slightly higher for killed reactors (Table 3) than for microalgae reactors (46% by day 2 and 60% by day 7).

Table 3

Table 4 shows the removal yields at the final time for the three pesticides. Removal from the liquid phase was calculated assuming that the pesticide was in the liquid phase initially, and the removal percentage was calculated based on the remaining pesticide in the liquid phase. The total degradation is the difference between the removal from the liquid phase and the sorption onto the solid phase. Photodegradation and other abiotic factors represent the pesticide removal percentage of the abiotic control, and biodegradation by the microalgae consortium was defined as the difference between total degradation and abiotic removal and other abiotic factors.

Table 4

As can be deduced from Figure 1 and is presented in Table 4, OXA showed the highest total degradation (photodegradation or another mechanism such as biodegradation) by day 7 (55.5 ± 15.6%) in comparison with CHL (35.4 ± 7.1%) and CYP (13.8 ± 2.7%). OXA removal increased with time from 41.0% at day 2 to 55.5% at day 7. In the case of CHL, the total degradation after

day 2 was higher (45.6 ± 10.6%) than the value at day 7, which could be attributable to pesticide adsorption on the glass flasks and its later desorption, as was reported previously by others authors considering hydrophobic pesticide removal [50]. The difference of pesticide degradation of CYP between day 2 (20.8 ± 15.2%) and day 7 (13.8 ± 2.7%) could be associated with the same cause. According to their removal, the theoretical half-lives of the pesticides in contact with microalgae biomass were 38 d for CYP (r^2 = 0.987), 13 d for CHL (r^2 = 0.925), and 5 d for OXA (r^2 = 0.804).

As shown in Table 4, removal from the liquid phase included the contribution of pesticide sorption to the solid phase plus the total pesticide degradation. In this sense, CHL exhibited the greatest removal from the aqueous media (97.3%) after 7 days of treatment with the microalgae consortium, followed by OXA (88.4%) and CYP (73.9%). Photodegradation was quantified by considering pesticide removal in the abiotic control (Table 3) to analyse the influence of abiotic processes on pesticide removal. CHL removal in the absence of the microalgae consortium (abiotic control) was 16.3 ± 4.5%, suggesting a slight influence of photolysis and other abiotic factors in pollutant removal. It has been reported that CHL has a limited potential for photodegradation by natural sunlight irradiation in water [51,52]. With regard to CYP, 27.1 ± 12.0% was removed from the abiotic control. For OXA, no contribution of abiotic factors was detected in the removal, suggesting that the pollutant elimination was influenced by neither photodegradation nor volatilisation. Moreover, it has been reported that OXA presents a medium to low volatility [27]. Bearing in mind that biodegradation was assumed to be the difference between total pesticide removal and photodegradation (based on Hom-Díaz [53]), it can be suggested that OXA removal can be mainly attributed to biodegradation (55.5 ± 15.6%) and sorption (32.8 ± 12.7%). Regarding CYP, biodegradation was not identified, and the main removal mechanisms seemed to be sorption (60.1 ± 11.1%) and photodegradation (27.1 ± 12.0%).

The results indicate that the total pesticide degradation in the microalgae reactors was higher for OXA, followed by CHL and CYP, respectively. Biodegradation by the microalgae consortium contributed to total degradation by 19.1% for CHL and 55.5% for OXA. Moreover, algal-mediated photolysis seemed to have an effect in CHL and CYP losses (16.3% and 27.1%, respectively), because oxygen and oxidant species generated by photosynthetic microorganisms are capable

of inducing indirect photolysis [54]. In the microalgae reactors, pesticide retention by sorption to the solid phase was similar for CHL and CYP (61.9% and 60.1%, respectively) but lower for OXA (32.8%). Overall, efficient removal from the liquid phase was accomplished for the three pesticides (CHL > OXA > CYP).

Given that the identification of TPs is directly associated with microalgae consortium activity, they were analysed in the microalgae reactors. As CHL has low solubility in water, its bioavailability for microbial degradation and its use as a carbon source could be limited [55,56]. However, O,O-diethyl thiophosphate (DETP) has been identified as a TP of CHL in microalgae reactors. The corresponding retention times, measured masses, molecular formulae (calculated on the basis of their accurate mass measurements and the observed isotopic patterns), relative mass measurement errors, and degree of unsaturation, expressed as ring and double bound equivalents, for this TP are summarised in Table 5. Higher intensities of this metabolite were observed at day 7, suggesting that it was produced gradually as degradation proceeded.

CHL degrading microorganisms are able to produce organophosphate hydrolysing enzymes that hydrolyse the P–O bond, leading to the corresponding dialkyl phosphate (DETP) and corresponding aryl alcohol (3,5,6-trichloro-2-pyridinol, known as TCP) [57]. In this study, DETP was identified as a TP of CHL by microalgae degradation. Studies examining CHL microbial degradation have focused mainly on bacteria and fungi [5,58–62], but few studies have assessed the capability of microalgae and cyanobacteria to degrade this compound. In general, it has been reported that degrading bacteria hydrolyse CHL, producing two main metabolites: DETP and TCP [56,63]. In this study, TCP was not identified, suggesting further conversion into other compounds or complete mineralisation, as Barathidasan et al. [64] found with a *Phanerochaete chrysosporium* fungal strain that could use TCP as a carbon source and completely mineralise CHL.

Table 5

In this work, no TPs were identified in association with OXA degradation, which could indicate that the molecule was mineralised. This assumption is coherent with the high biodegradation detected (55.5%). Some authors have demonstrated an ability of other microorganisms to biodegrade OXA. For instance, *Pseudomonas fluorescens* CG5 isolated from a soil contaminated with herbicides was able to use OXA as a carbon source and further catabolise the compound, obtaining a few toxic metabolites such as indole, benzoic acid, and trimethyl benzene, including dehalogenation [25].

Most of the published studies regarding CYP biodegradation are focused on bacteria and fungi [65–68] and not on microalgae. In general, it has been reported that hydrolysis of the ester bond is the main degradation pathway of CYP, producing the corresponding alcohol (2-hydroxy-2(3-phenoxyphenyl)acetonitrile, known as CPBA) and acid (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylic acid, known as DCCA) [2]. CPBA is further transformed, leading to small molecular weight aliphatic compounds with intermediate compounds such as 3-phenoxybenzoic acid (PBA) [2]. These aliphatic compounds include oleic acid [1,2], 2-pentadecanone [2], acetic acid [4], decanoic acid [4], 1-dodecanol [1], and isopropyl myristate [1]. Likewise, phototransformation products of CYP have also been reported previously, with DCCA and PBA being the main photoproducts [69]. In this work, no TPs related to microalgae biodegradation of CYP were identified. This result is consistent with the low observed total degradation (13.8%), which indicates that the main removal mechanism of CYP in the liquid phase is sorption onto the microalgae. This low degradation yield probably led to poor formation of TPs and hence no detection.

The physiological status of microalgae is in concordance with biomass growth, and cell dry weight is considered an integral parameter of cellular metabolism [70]. Thus, the evolution of biomass concentration in microalgae reactors could be an indicator of the toxicity and inhibition caused by the pesticide. As shown in Figure 2, the biomass concentration in the microalgae reactors

415 increased by 8%, 19%, and 15% for CHL, CYP, and OXA after 7 days, suggesting that these
416 molecules were not toxic to the microalgae consortium. Moreover, no statistical differences were
417 identified between the mean values of biomass in blank and microalgae reactors for each
418 pesticide at each sampling time (p > 0.05, except for OXA at time 0). As shown in Fig. 2, the
419 pesticide concentration was not toxic for the microalgae consortium. On the other hand,
420 ecotoxicological data indicate that the acute 72-h EC_{50} values of CHL, CYP, and OXA are 0.48
421 mg L^{-1} (for unknown microalgae species), > 0.0667 mg L^{-1} (for *Pseudokirchneriella subcapitata*),
422 and 0.004 mg L^{-1} (for *Scenedemus subspicatus*), respectively [71]. Bearing in mind that these
423 toxicological studies were performed using pure microalgae species, it is not possible to relate
424 them with the microalgae consortium employed in the present work. The advantages of working
425 with a microalgae consortium are the synergetic interactions between microorganisms that
426 enhance the robustness of the system and overall removal efficiency [17,39,72,73].

427 Figure 2

428 (one-column fitting image)

429

430 **3.2. Anaerobic treatment and methane production**

431 Anaerobic digestion has been proposed as a technique for microalgae biomass treatment and
432 valorisation after the aerobic phase [74,75]. The anaerobic biodegradation and the biogas
433 production potential of the microalgae biomass containing the retained pesticide by sorption was
434 assessed by BMP test. During anaerobic digestion processes (hydrolysis, acidification, and
435 methanisation), pesticides and other compounds can be physically, chemically, and biologically
436 transformed [76] by diverse microorganisms and enzymes involved in each phase. Given the high
437 sorption affinity of the studied pesticides, their concentration in BMP tests was determined in the
438 biomass at the initial (t_0) and final time (t_{42}).

439 The net methane production (Fig. 3) was better for BMP reactors containing CYP and CHL, with
440 yields of 4880 and 4558 NmL CH_4 g VS^{-1}, respectively (differences were not statistically
441 significant). The methane yield in flasks containing OXA was remarkably lower (2919 NmL CH_4 g
442 VS^{-1}, p < 0.05). According to the Gompertz model, the lag phase was around 2.9 to 3.5 days

443 (Table 6), after which biogas production increased sharply at a rate of 27.4, 24.3, and 21.8 mL
444 CH_4 d^{-1}. VFAs quantified after the methanisation verified the stability of the process (Table 6),
445 indicating neither inhibition from the residual pesticide nor the potential TPs.

446 Analysis of the final digestate indicated that CHL and CYP were efficiently removed by 87.4 ±
447 0.5% and 58.7 ± 3.9%, respectively (difference of the mean pesticide content in the solid phase
448 between the initial time and final time was statistically significant in both cases, $p < 0.01$) (Fig. 4).
449 This is in agreement with the work of Lian et al. [77], in which contaminated biomass with the
450 insecticides parathion and malathion (organophosphorus compounds) was efficiently
451 anaerobically transformed by reduction reactions and enzymatic hydrolysis driven by hydrolases,
452 suggesting that hydrolytic activities provide a potential tool for biodegradation of
453 organophosphorus compounds such as CHL. Likewise, García-Mancha et al. [78] reported a
454 good removal efficiency (77%) of CHL from wastewater under thermophilic conditions (55 °C).
455 The results indicate that although OXA was highly degraded by the microalgae consortium under
456 aerobic conditions (55% biodegradation), its anaerobic removal was lower (18.7 ± 4.4%, $p < 0.05$)
457 (Fig. 4), leading to a minor methane yield. Otherwise, CHL and CYP were successfully degraded
458 by the anaerobic microorganisms as confirmed by their removal in the digestate. The results
459 indicate that anaerobic degradation of CYP was more effective than aerobic degradation with the
460 microalgae consortium.

461 Figure 3

462 (one-column fitting image)

463

464 Figure 4

465 (one-column fitting image)

466

467 Table 6

468

After the aerobic treatment, water could be treated in a municipal WWTP or discharged if adequate parameters were fulfilled. Moreover, the solid phase containing pesticides could be valorised as proposed in this work through methanisation for biomethane production. This suggestion is in agreement with other studies [79–81]. Even though anaerobic digestion contributed to the elimination of pesticides, the digestate obtained after the anaerobic digestion could be dewatered. The solid fraction could be employed for fertilisation [82], and the water could be treated in a WWTP or properly discharged if parameters were adequate given environmental restrictions.

4. Conclusions

Phycoremediation coupled with anaerobic degradation of contaminated biomass was assessed for hydrophobic pesticides removal. The distribution of pesticides on both liquid and solid matrices was evaluated to determine the capacity of a microalgae consortium to degrade complex molecules. Total degradation (biodegradation and photodegradation) was higher for OXA (55%), followed by CHL (35%) and CYP (14%); whereas CHL and CYP exhibited greater sorption to the solid phase (62% and 60%, respectively). Overall, a large removal from the liquid phase (total degradation + sorption) was achieved for the target compounds (CHL: 97%, CYP: 74%, and OXA: 88%). The results evidence the potential of algae-based bioremediation technologies in the bioconversion of agrochemicals. Further anaerobic degradation of the biomass containing the target pesticides was performed without inhibition and led to high methane generation and removal of the pesticides (removal efficiency was CHL > CYP > OXA). Based on this study, microalgae biomass containing pesticides could be valorised efficiently by anaerobic digestion, fostering biogas production.

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500

501 **Competing interest statement**

502 We declare that no conflict of interest exists in the submission of this manuscript.

503

504 **CRediT author statement**

505 Romina Avila: Research, Writing: original draft. Andrea Peris: Pesticides analysis and TPs
506 identification. Ethel Eljarrat: Methodology, Supervision, Writing: review and editing. Paqui
507 Blánquez and Teresa Vicent: Methodology, Supervision, Writing: review and editing.

508

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