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Green alga *Chlamydomonas reinhardtii* can effectively remove diclofenac from the water environment – A new perspective on biotransformation

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HIGHLIGHTS

- *Chlamydomonas reinhardtii* effectively removes diclofenac (DCF) from the aqueous solution.
- *C. reinhardtii* uses various mechanisms to reduce the level of DCF.
- DCF removal is based on sorption and biotransformation.
- DCF metabolites of phase I and II in *C. reinhardtii* cultures were identified.

GRAPHICAL ABSTRACT

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ABSTRACT

The use of unicellular algae to remove xenobiotics (including drugs) from wastewaters is one of the rapidly developing areas of environmental protection. Numerous data indicate that for efficient phycoremediation three processes are important, i.e. biosorption, bioaccumulation, and biotransformation. Although biosorption and bioaccumulation do not raise any serious doubts, biotransformation is more problematic since its products can be potentially more toxic than the parent compounds posing a threat to organisms living in a given environment, including organisms that made this transformation. Thus, two questions need to be answered before the proper algae strain is chosen for phycoremediation, namely what metabolites are produced during biotransformation, and how resistant is the analyzed strain to a mixture of parent compound and metabolites that appear over the

Abbreviations: CYP450, cytochrome P450; DAD, diode array detector; DCF, diclofenac; DMSO, dimethylsulfoxide; ECx, effective concentration (a concentration that caused x % inhibition of population/organism growth); ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSM, Sueoka's High-Salt medium; LLE, liquid-liquid extraction; NSAIDs, non-steroidal anti-inflammatory drugs; PPCPs, pharmaceuticals and personal care products; QY, quantum yield; SE, standard error; SPE, solid phase extraction; TPs, transformation products; USE, ultrasonic extraction.

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course of culture? In this work, we evaluated the remediation potential of the model green alga *Chlamydomonas reinhardtii* in relation to non-steroidal anti-inflammatory drugs (NSAIDs), as exemplified by diclofenac. To achieve this, we analysed the susceptibility of *C. reinhardtii* to diclofenac as well as its capability to biosorption, bioaccumulation, and biotransformation of the drug. We have found that even at a relatively high concentration of diclofenac the algae maintained their vitality and were able to remove (37.7%) DCF from the environment. A wide range of phase I and II metabolites of diclofenac (38 transformation products) was discovered, with many of them characteristic rather for animal and bacterial biochemical pathways than for plant metabolism. Due to such a large number of detected products, 18 of which were not previously reported, the proposed scheme of diclofenac transformation by *C. reinhardtii* not only significantly contributes to broadening the knowledge in this field, but also allows to suggest possible pathways of degradation of xenobiotics with a similar structure. It is worth pointing out that a decrease in the level of diclofenac in the media observed in this study cannot be fully explained by biotransformation (8.4%). The mass balance analysis indicates that other processes (total 22%), such as biosorption, a non-extractable residue formation, or complete decomposition in metabolic cycles can be involved in the diclofenac disappearance, and those findings open the prospects of further research.

1. Introduction

The constantly growing concentration of anthropogenic contaminants in natural water bodies is a very important problem of our times. Pharmaceuticals, among them non-steroidal anti-inflammatory drugs (NSAIDs), belong to so-called micropollutants or contaminants of emerging concern; found in the environment at a relatively low concentrations yet they cause significant threat to the non-target organisms [1]. NSAIDs can disrupt crucial metabolic pathways in both animal and plant cells, causing their dysfunction, developmental disorders, or even death [2,3]. Besides the acute toxic effects, NSAIDs are known to cause long-term (chronic) effects because of their abilities to bioaccumulate and/or bioconcentrate in organisms [4]. It is further complicated by the fact that in the natural environment NSAIDs can undergo physico-chemical transformation (e.g. photolysis, oxidation, complexation) and biotransformation (metabolic transformations) [5,6], which leads to the formation of mixtures of intact drugs and their numerous derivatives. Toxic effects of such mixtures are almost unpredictable [5] thus NSAIDs removal from natural water bodies as well as wastewaters is a matter of high priority [1,7].

Among NSAIDs detected in samples derived from seas, inland water bodies, groundwaters and even drinking water sources, diclofenac (DCF) is one of the most often reported [8]. The main DCF sources are human and veterinary drugs, thus intact DCF and its derivatives enter the water environment *via* landfill leachate and municipal and industrial wastewater [9]. The concentrations of DCF in surface waters, sewage and drinking/groundwater are reported in a wide range depending on the location, up to 40,570 ng/L [10]. Although hazardous contaminants are supposed to be removed from landfill leachate and wastewater before they reach the environment, conventional leachate-treatment procedures and wastewater treatment plants remove organic substances, including drugs like DCF, with varying efficiency [11,12]. Therefore, continuous efforts are made to develop new procedures and techniques to prevent the leakage of DCF and other NSAIDs into the environment. Among conventional methods used to eliminate DCF from the aquatic environment, sedimentation, precipitation, membrane filtration, ozonation, chemical oxidation, Fenton oxidation, electrochemical oxidation, photocatalysis, and adsorption on activated carbon are reported [13]. To enhance the efficiency of removing DCF from the treated water, physical and chemical methods are often combined with biological methods in which bacterial consortia are used for biotransformation and/or biodegradation of drug residues that remained after the physico-chemical phase of water treatment [6,14]. In recent years, increased attention is paid to algae as a possible biocomponent of water treatment systems because of their bioremediation (biosorption, bioaccumulation and/or biodegradation) potential [15–20]. The advantage of using algae may be that it can simultaneously remove inorganic pollutants (such as nitrogen, phosphorus and metal ions) and organic pollutants (such as pharmaceuticals and personal care products (PPCPs)), and both types of pollutants in small doses can have a stimulating effect on algae growth [15]. Microalgae's PPCPs removal efficiency can be higher than conventional wastewater treatment, and when using algae in hybrid systems, removal efficiency can exceed 97% [7,18,21]. The maximum DCF removal rate varies by algal species and ranges from 22% for *Chlorella vulgaris* to 79% for *Scenedesmus obliquus* [10]. The high bioadsorption and bioaccumulation capacity of *Chlamydomonas reinhardtii* has been shown for various PPCPs [7]. What is interesting, *C. reinhardtii* is able to transform xenobiotics through various enzymatic reactions such as hydrolysis, CYP450 oxidation reactions, methylation and glutamate conjugation [17]. Studies by Zhou et al. have shown that *Chlamydomonas reinhardtii* is more effective in removing DCF from wastewater compared to other green microalgae [15]. In comparison to other green algae *Desmodesmus subspicatus* (EC_{50} = 72 mg/L) [22], *Scenedesmus vacuolatus* (EC_{50} = 23 mg/L) [23] or higher plants such as *Typha latifolia* (stress-inducing concentration = 1 mg/L) [24] or *Lemna minor* (growth-inhibiting concentration = 100 μg/L) [25,26], *C. reinhardtii* is characterized by low sensitivity to DCF and therefore is a promising organism for phytoremediation models. The previously determined $EC_{50/24}$ of DCF (a concentration that caused 50% inhibition of population growth after 24 h of exposure) for *C. reinhardtii* is quite high and reaches 135.5 mg/L [27]. At this concentration numerous morphological, physiological, and biochemical abnormalities have been described [28–30], such as photosynthesis inhibition (by about 30–50%), chlorophylls content decrease (about 40–50%), H_2O_2 overproduction (about 200% of control), changes in pattern of antioxidative-enzymes-encoding genes and increase in these enzymes activity, cells enlargement and deformation etc. However, at a DCF concentration corresponding to $EC_{25/24}$ (65.75 mg/L), the content of total RNA, protein, and chlorophyll *a* did not differ statistically in the treated and control cells [28]. In this work, it was decided to use a concentration corresponding to toxicological value $EC_{10/24}$, since this concentration will not cause such strong damage to the vital functions of the cell as the concentration corresponding to $EC_{50/24}$, but at the same time, it is much higher than that observed in natural and wastewaters (0.5–40,570 ng/L) [10]. Thus, the chosen DCF concentration should not inhibit the main physiological processes in the cell, but trigger the protective mechanisms and enable cells to biotransform DCF. In addition, in the study of long-term action of xenobiotics, using relatively high concentrations of DCF reduces the risk of a decrease in its level in the culture medium during the experiment and thus does not require the use of complex flow-through systems [31]. Thus, despite the concentration chosen by us is much higher than the concentrations detected in water reservoirs, we assume that if algae grown in optimal laboratory conditions can maintain vitality and metabolize DCF at a concentration corresponding to EC_{10}, then a similar phenomenon should occur in an environment where concentrations of the toxicants are lower, but the growth conditions are less favorable.

In the present study complex research was conducted to evaluate the usefulness of green alga *C. reinhardtii* for DCF removal from the water environment and to assess the contribution of biosorption and

biotransformation processes to the drug elimination. Our study focused on the identification of the DCF transformation products (TPs) in both biomass and culture media. We intentionally use the term: transformation products, because the design of the experiment does not allow for a definite conclusion that all of the identified substances are formed with the *C. reinhardtii* enzymatic system. Since DCF and its TPs may cause diverse effects on non-target organisms, the study of the biotransformation of DCF by aquatic plants may be the first stage of further study of the toxicity of these compounds [32]. In this regard, another important aspect of this work was the elucidation of the structure of DCF TPs to propose a pathway for DCF degradation in *C. reinhardtii* cultures.

2. Materials and methods

2.1. Culture growth conditions and exposure to the toxicant

Cultures of *C. reinhardtii* 21 gr (CC–1690, wild type; Chlamydomonas Resource Center, http://www.chlamy.org) were grown aseptically in glass vessels in a mineral medium (HSM) [33], at constant conditions: 30 ºC, with air-CO_2 (2% v/v) aeration under constant illumination (150 μmol·m^{-2}·s^{-1}) of photosynthetically active radiation (graphic design of the experiment is provided in the S1 Fig). Before each experiment, the cultures were diluted with sterile HSM to a starting density of $4.6 \times 10^5 \pm 0.1 \times 10^5$ cells/mL and divided into three sub-populations: control (C1), control with DMSO (C2), and DCF-treated variants (DCF). Each experiment lasted 4 days.

Diclofenac sodium salt (CAS 15307–79–6, Sigma–Aldrich,) was dissolved in dimethylsulfoxide (DMSO) and added to HSM to obtain a final concentration of 32.7 mg/L, corresponding to the $EC_{10/24}$ [27]. The final DMSO concentration in culture media for C2 and DCF variants was ≤ 0.1% (v/v). Cultures were sampled every day of the experiment for further analyses. Based on the fact that DCF can decompose under the influence of visible light [34,35] with the formation of toxic products [23] several additional experimental variants, containing HSM with DCF at the same concentration but without algae were prepared, to assess the effects of potential confounding factors, e.g. presence of DMSO in culture media and aeration (S1 Fig). Appropriate HSM controls without algae but spiked with DCF were prepared and kept under light exposure (HSM A–C) and in a dark (HSM D) to monitor the removal of DCF by photolysis (S1 Fig). All experiments were performed in triplicate under sterile conditions.

2.2. Determination of neutral lipids content

The neutral lipids content was determined daily using the microplate-based method with Nile Red (BioReagent, Sigma–Aldrich, Germany) staining [36]. For analysis, 100 μL of cells suspension was mixed with 5 μL of dye solution (0.5 mg/mL DMSO), after 20 min incubation in the dark, the fluorescence intensity (Ex/Em: 485/595 nm) was measured by microplate-reader (Tecan Infinite F200 Fluorescence Microplate Reader, Tecan Group Ltd., Männedorf, Switzerland). Lipid concentration was extrapolated from the intensity of the signal obtained for the 6-point curve for triolein at a concentration of 1.62–26.04 μg/mL and expressed in picograms per cell.

2.3. Chlorophyll a fluorescence in vivo

Chlorophyll *a* fluorescence *in vivo* was assessed by measuring and analyzing OJIP curves using an AquaPen-C100 (Photon Systems Instruments, Drasov, Czech Republic) run by the FluorPen 1.1.2.3 software [37]. The QY was defined as the ratio of the number of photons emitted to the number of photons absorbed by the sample.

2.4. Cells number

Cells number was estimated with the Multisizer 4 (Beckman Coulter) in glutaraldehyde-fixed samples (final glutaraldehyde concentration 0.2% v/v) by diluting 50 μL of fixed sample in 10 mL 0.9% NaCl. The results were given as cells number per mL of the culture. Based on the number of cells, the doubling time and the growth rate of the population were calculated. The growth rate was calculated using the formula: $K = (\ln(N_t)–\ln(N_0))$/day, where N_t – the number of cells at a time point, N_0 – the initial number of cells.

2.5. Determination of singlet oxygen in the cell

Intracellular singlet oxygen fluorimetric assay for *C. reinhardtii* cells was performed using a Singlet Oxygen Sensor Green ™ [38]. For this, 5 μL of the Singlet Oxygen Sensor Green ™ working solution (0.1 mM in methanol) and cell culture (100 μL) were added to the well of a 96-well plate. An appropriate volume of distilled water was added to control wells to serve as a blank. The fluorescence intensity (Ex/Em = 485/535 nm) was measured by microplate-reader (Tecan Infinite F200 Fluorescence Microplate Reader, Tecan Group Ltd., Männedorf, Switzerland) immediately after sample preparation. The received signal intensity was expressed in arbitrary units recalculated per one cell.

2.6. Sample preparation and diclofenac extraction

The cell suspension was sampled from culture vessels daily and volumes ranged from 200 mL to 800 mL to obtain 100–200 mg of dry biomass were collected. Samples were centrifuged at 3784 x *g* for 5 min, supernatant was collected, and the pellet was washed 2 times with 50 mL of pure HSM, freeze-dried (Scanvac CoolSafe, Labogene, COUNTRY), and stored at − 80 °C. Each washing was also collected and the DCF content determined to estimate the adsorption of DCF loosely bound to the cell walls. Culture medium samples (supernatant obtained after centrifugation and biomass separation and HSM obtained after pellet washing) and control media samples without algae were processed as follows: 50 mL of medium was freeze-dried and the final residue was dissolved in 1 mL of methanol and filtered through 0.2 μm sterile membrane filter for HPLC analysis. Extraction of DCF from biomass samples was performed using ultrasonic extraction (USE) followed by solid phase extraction (SPE).

2.6.1. Ultrasonic extraction (USE) of diclofenac and its metabolites from biomass

The extraction of dry biomass was carried out in 10 mL glass test tubes with a mixture of acetone/methanol (1/1, v/v) at the ratio of 10 mL per 100 mg of biomass. For 100 mg of dry biomass 5 mL of the solvent mixture was added, then the tubes were vortexed (1 min) and left in an ultrasonic bath with an ultrasonic frequency of 25–40 kHz for 15 min at room temperature. After that, the extract was centrifuged at 4 000 g for 10 min, the supernatant was collected, and the remaining pellet underwent the second extraction with another 5 mL of solvent. The combined extracts (total about 10 mL) were diluted with deionized water (up to 5% volume) for subsequent solid-phase extraction (SPE). To monitor the stability of DCF during sample preparation, the stock solution of DCF was treated in the same manner as real samples.

2.6.2. Solid-phase extraction (SPE)

SPE was used to clean up and concentrate the biomass samples, which is especially important due to the large amount of pigments contained in the biomass. A CHROMABOND® SPE vacuum manifold for 12 cartridges (Macherey-Nagel, Duren, Germany) was used with SPE Oasis HLB 6cc/150 mg cartridges (Waters, Milford, MA, USA) containing hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene, which showed high recovery of DCF when extracted from plant samples [39,40]. The cartridges were preconditioned sequentially with 1 mL of

methanol and 1 mL of ultrapure water. After that, the diluted extracts (about 200 mL) were loaded onto cartridges and dried under nitrogen. For the complete elution of compounds of interest, 2 × 1 mL of methanol was used. Then the eluates were dried under nitrogen and resuspended in 150 μL of methanol for further high-performance liquid chromatography-diode array detection-electrospray ionization-high resolution mass spectrometry (HPLC/DAD-ESI-HRMS) analysis.

2.7. HPLC/DAD-ESI-HRMS analysis

DCF and DCF TPs and their relative content were identified and quantified using high-resolution mass spectrometry (HRMS). The extracts were analyzed using a Dionex UltiMate 3000 HPLC system (Thermo Scientific,) coupled with a diode array detector (DAD at a monitoring wavelength of 280 nm) and with electrospray ionization sourced-high resolution mass spectrometer (ESI-HRMS; Impact HD Mass Spectrometer, Bruker,). Separation was achieved on a reversed-phase column (Phenomenex Kinetex C18 column, 150 × 4.6 mm, 2.6 μm), held at 30 °C using a standard analytical gradient (50–100% MeOH (Sigma–Aldrich, LC-MS grade) for 20 min, kept 100% for 5 min) at a constant flow rate of 0.6 mL/min.

2.8. Operational settings of the high-resolution mass spectrometer and DCF TPs identification

Following operational settings of the high-resolution mass spectrometer (Bruker Impact HD) were used: dry temperature 200ºC; drying gas flow 12 L/min; nebulizer 3 bar; capillary voltage 4500 V; endplate offset 500 V. The spectra were collected in the positive ion mode in the range of 200–500 *m/z* with spectra rate 2 Hz. The CID was set as a ramp from 20 to 60 eV on masses 200–1200, respectively. Collision energy 20 eV (found as the optimal fragmentation energy of DCF ions and its metabolites). Calibration was performed using CH_3COONa clusters at the beginning of each analytical batch.

DCF concentration was determined using a 6-point calibration curve in the range of 1–50 μg/mL). Since standards for most DCF metabolites were not commercially available, a semi-quantitative mass spectrometry approach based on a comparison of peak areas was used to determine the levels of DCF TPs in biomass and the medium. This strategy is based on the structural similarity between the parent compound and their TPs and the respectively similar ratio of the peak area and the concentration (response factor) [41]. Based on equation C(TP) = peak area(TP)/response factor (parent compound), percentage ratio of TPs to the level of DCF concentration (total amount) was calculated as a percentage ratio of their peak areas [41]. Removal of diclofenac was calculated as a ratio between its concentration measured in the nutrient medium at the end of experiment and the initial concentration, and was expressed in percents.

To determine the amount of DCF adsorbed on the cell surface, the content of DCF in the nutrient medium after the first and second biomass washes was calculated and summarized at all time points. The amount of DCF adsorbed on the surface of the culture vessels was calculated in a similar way, but instead of the nutrient medium, the level of DCF was determined in methanol after washing the culture vessels on the last day of the experiment. The total intracellular (absorbed) DCF content was calculated as the sum of DCF content in the collected biomass at all time points of the experiment.

For non-targeted screening of organohalogenated chemicals in complex matrices, the HaloSeeker 1.0 application was used aimed at searching for halogenated signatures in full scan HRMS fingerprints [42]. In addition, a manual search for Cl-containing DCF metabolites was performed on the chromatograms according to a characteristic isotopic pattern, which was then checked using the online tool “Isotope Distribution Calculator and Mass Spec Plotter” (Scientific Instrument Services by Adaptas Solutions, Palmer, MA, USA) available at https://www.sisweb.com/mstools/isotope.htm). Bruker Compass DataAnalysis (SmartFormula Manually command) was used to calculate the possible molecular formula for a given *m/z* value for candidate structures and report the error of mass accuracy (deviation between measured mass and theoretical mass of the selected peak in part per million (ppm)), and rings plus double bonds (RDB) values. To elucidate the structures of candidate metabolites, fragmentation and neutral loss spectra were used, which were obtained using LC-QqQ-MS in the product ion scan and neutral loss scan modes. To further filter isomers of potential metabolites, octanol-water partition coefficients (logP) were calculated (ACD/ChemSketch, version 2020.2.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2022.), which were used to predict theoretical retention times and compare them with real values [43]. In addition, in silico biotransformation modeling using SMARTCyp [44], XenoSite P450 [45], and CYPstrate [46] software was used to predict CYP mediated phase I metabolism of DCF (prediction of the sites of the molecules that are most susceptible to metabolism mediated by CYP450.

2.9. Statistical analysis

Statistical analysis was performed using MS Excel 2010 (Microsoft) and Statistica 13.0 (TIBCO Software Inc. (2017),). All data were expressed as the mean of at least three independent experiments ± SE (standard error). The same software was applied to compute a basic statistic and the nonparametric Mann-Whitney U test for the population could not be assumed to be normally distributed. A p-value < 0.05 was considered significant.

3. Results

3.1. Algae response to diclofenac

The growth pattern of control (non-exposed cultures, described as C1) was typical for the exponentially growing microalgae culture; the growth rate (K) was the highest at the beginning of the experiment (K=1.77 day^{-1}) and gradually decreased along with increasing population density (S2 Fig). At the end of the experiment, the growth rate decreased to 0.69 day^{-1}. No significant differences were found in the growth rate between control (C1) and DMSO-treated cultures (described as C2). In contrast, the statistically non-significant growth-inhibiting effect of DCF could be observed from the beginning of the experiment, and after 72 h and 96 h the growth rate constant (K) was significantly lower as compared to control C1, by 31% and 37%, respectively.

It is worth noting that at 72 and 96 h, significant differences in the pH values were observed between experimental variants (data not shown). While in the controls the pH decreased from 7.4 to 4.6 for C1 and from 7.4 to 5.4 for C2 during the experiment, the decrease in pH reached only 6.1 in the cultures exposed to DCF. These differences could be explained by limited buffering capacity of HSM; since intensive uptake of most cationic nutrients (mainly NH_4^+) is coupled to H^+ secretion into the medium, a significant drop in pH value is often observed in the cultures with high rate of biomass accumulation [47].

The relative level of singlet oxygen in control (C1) at the beginning of the experiment was 0.0022 a.u. per cell and dropped to 0.0004–0.0006 a.u. per cell during the course of the experiment. We showed that the relative level of singlet oxygen decreased in C2 cells at 24 h and increased at 72 h and 96 h compared to C1 cells. In DCF-treated algae, the relative level of singlet oxygen increased by 292% and 791% when compared to C1 cells after 72 and 96 h, respectively, and increased by 83%, 148%, and 358% when compared to C2 cells after 48, 72, and 96 h (S3 Fig).

The neutral lipids content in control cells (C1) ranged from 0.23 pg per cell at the start of the experiment to 3.39 pg per cell at the end of the experiment. We observed a decrease in neutral lipids level at 24 h and an increase at 72 h and 96 h in C2 cells compared to C1. In DCF-treated algae, after 24 h and 96 h, we noted a decrease in neutral lipids level

by 33% and 84% compared to control C1, and a decrease by 48% and 85% compared to control C2 after 72 h and 96 h, respectively (S4 Fig).

The photosynthesis efficiency, estimated from the measurement of chlorophyll *a* fluorescence *in vivo*, remained stable during the experiment for both C1 and C2 algae, and relative quantum yield parameter (QY) value oscillated around ~0.7. We noticed a significant decrease in QY value in DCF-treated algae starting from 48 h of the experiment, where QY was diminished by 25–42% as compared to C1, and by 13–85% compared to C2 (S5 Fig).

3.2. Kinetics of DCF biodegradation (DCF Removal by C. reinhardtii)

In all algae-free controls containing DCF (HSM B-E), its concentrations did not change till the end of experiment (96 h), and were 29.8 ± 0.15 mg/L for HSM B, 28.7 ± 0.11 mg/L for HSM C, 29.2 ± 0.10 mg/L for HSM D, and 28.6 ± 0.15 mg/L for HSM E. Additionally, DCF was undetectable in the non-exposed algae (C1 and C2) and media blanks (HSM A and HSM F). In the presence of *C. reinhardtii* (Fig. 1) the concentration of DCF in medium decreased by about 37.7% (Fig. 1A). The content of DCF in the biomass gradually increased throughout the experiment and at 96 h reached 2.86 ± 0.51 mg/g of dry mass (Fig. 1B).

3.3. Identification of DCF transformation products

HRMS data were used for the identification of DCF transformation products (TPs) and their identification was carried out using tiered approach.

Firstly, stock solution of DCF was screened for potential DCF related impurities that might further interfere with the identification algae specific TPs. A compound with *m/z* 313.9895 which corresponds to the addition of one chlorine atom to the DCF molecule ($C_{14}H_{11}Cl_3NO$) was present in a stock solution and all experimental samples (controls and exposed to DCF). At the same time, its concentration, as well as the concentration of DCF, decreased in the medium during the experiment. The presence of this compound in the standard stock solution made it possible to consider it as a DCF impurity [48,49].

Next, the available literature data on the metabolism of DCF by various organisms was reviewed to list the structures already confirmed as its TPs (S1 Table). HRMS generated spectral data were filtered for those present in DCF exposed cultures (biomass and media). A non-targeted screening of organohalogen compounds (HaloSeeker 1.0), calculation of isotope distribution and possible molecular formula (Isotope Distribution Calculator and Mass Spec Plotter (Scientific Instrument Services by Adaptas Solutions, Palmer, MA, USA) and Bruker Compass DataAnalysis (SmartFormula Manually command)), as well as *in silico* prediction of CYP-mediated phase I DCF metabolism (SMARTCyp, XenoSite P450 and CYPstrate) were performed (for more details, see Materials and Methods).

Furthermore, the formation of DCF degradants under ultrasonic irradiation [50] or the formation of an artifact of DCF (1-(2,6-dichlorophenyl)indolin-2-one) during extraction [51] was assessed by the ultrasonic extraction of the stock solution of DCF. To avoid false positive detections being a result of presence of impurities in commercial DCF sodium standard, stock solution was also monitored with LC-HRMS [52]. None of the above compounds were found (except to aboved impurity with *m/z* 313.9895) in the stock solution of DCF.

None of the TPs were detected in any algae-free controls (HSM A-F), except for 8-chlorocarbazole-1-yl-ethanoic acid (DCF_M260) [53], the well-known photodegradation product of DCF, which is characterized by the loss of one chlorine atom and was detected only in the algae-free control exposed to light (HSM B and HSM C) and DCF-treated algae. Although efficient DCF photodegradation under visible light requires catalysts and/or bias voltage within a strictly defined range, non-catalyzed VIS-photolysis is reported to remove about 2–37% of the drug depending on the time and irradiation intensity [34,35,54–56]. Thus, despite we used the UV-free fluorescent lamps (OSRAM DULUX L55 W/950 Daylight, Milano, Italy) and the UV-impermeable glass culture vessels, the formation of small amount of DCF photodegradation products during long-term exposure was expected. Nevertheless, the process of photodegradation did not lead to a significant decrease in the level of DCF throughout the entire duration of the experiment (Fig. 1A); after 96 h of exposure the percentage ratio of the DCF_M260 peak area to the DCF peak area was only 1.34 ± 0.11%. At the same time no differences in the levels of DCF in HSM controls (HSM B–E) were observed in the dark and in the light. Except for the compounds listed above, 39 DCF derived TPs were identified in the samples of the nutrient medium and biomass, among which 17 were found for the first time (S1 Table).

Under the positive ionization, DCF decomposes while forming 4 main in-source fragments (*m/z* 278; 250; 215; 180) corresponding to the loss of water, carbon dioxide and chlorine atoms, respectively. A similar fragmentation pattern was observed in many DCF metabolites, which facilitated their identification.

Compounds DCF_M312A and DCF_M312B (*m/z* 312.0187) with retention times 11.8 and 12.3 min were identified as 5-hydroxy-diclofenac and 4′-hydroxy-diclofenac respectively based on the presence of a fragment ion which corresponds to the loss of water, as well as by matching the existing record in the massbank database. Although at different collision energies (20, 45, and 125 eV) there were no characteristic fragments of 5-hydroxy-diclofenac confirming the hydroxylation occurring in the non-chlorinated ring. The calculated LogP value for 5-hydroxy-diclofenac is lower (3.91) than that of 4′-hydroxy-diclofenac (4.56) and thus shorter retention time of metabolite DCF_M312A is expected. It should be noted, that in some studies the retention time of 4′-hydroxy-diclofenac is shorter than that of 5-hydroxy-diclofenac [57–59], but in almost all cases the level of 4′-hydroxy-diclofenac is noticeably higher. So, based on the above, the fact that the DCF_M312B is 4′-hydroxy-diclofenac can be confirmed by its peak area, which is 4 times larger compared to DCF_M312A, since 4-hydroxy-diclofenac is the main metabolite produced by most organisms and its amount will be greater than that of other isomers [19,60–63].

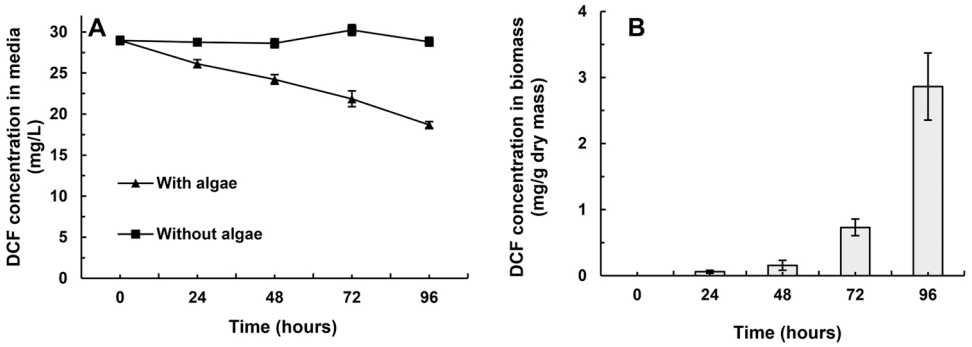

Fig. 1. The concentration of DCF in the media (A) and *C. reinhardtii* biomass (B) during the experiment.

Compound DCF_M310.04 was identified as diclofenac methyl ester (methyl DCF) by the characteristic fragment *m/z* 278 corresponding to the loss of -CH_4O, as well as by high similarity with the library spectrum (NIST, National Institute of Standards and Technology - MS number 120343).

The DCF_M278A corresponds to the loss of the water from DCF molecule and subsequent cyclization resulting in 1-(2,6-dichlorophenyl)− 2-indolinone. Also, for this metabolite, the spectrum was found to well match that in the library (MassBank Record: LU115002). Observed loss of -CO and multiple losses of -Cl is a typical fragmentation pattern for DCF transformation products.

DCF_M326 is characterized by the presence of fragments with *m/z* 266 and 230 characteristic for 4-hydroxy-diclofenac and a fragment of *m/z* 294 corresponding to the loss of -CH_4O suggests that this is a hydroxylated derivative of methyl-DCF. In addition, matches were found with the spectrum of this transformation product (SpectraBase Compound ID AGE2S6r9Nso).

DCF_M310.004 was previously described in [64] and identified as 5-hydroxy-diclofenac quinone imine based on the characteristic fragmentation pattern and also a retention time close to 5-hydroxy-diclofenac, which is explained by the fact that it is a direct product of the transformation of the last one with a similar structure. The structure of metabolite DCF_M266A was proposed based on the fragmentation pattern and the similarity of its fragments with DCF_M312A, which lost the -COOH group. The characterization of DCF_M266B was based on the presence of the *m/z* 202 fragment (loss of -CO), and on the calculated LogP value (5.04) which more corresponded to the retention time (16.4 min) compared to other possible structures. Fragments of *m/z* 214 and 248 identical with DCF_M250 and DCF_M264, respectively, may indicate that DCF_M266B is the product of their transformation.

Structure of DCF_M298B was proposed on the basis of a characteristic fragmentation pattern (loss of water and –CO), as well as on literature data [65], however, insufficient fragmentation data did not allow to establish in which position of the ring (4`- or 5-) the hydroxylation occurred. DCF_M298A has a shorter retention time than DCF_M298B and is not an isomer because it has a non-identical fragmentation pattern (no -CO loss), which may indicate that it is formed as a result of further hydroxylation of DCF_M282A.

DCF_M354 was identified as aceclofenac by comparison of MS/MS spectra with published spectra [66] (same fragmentation pattern with characteristic product ions *m/z* corresponding to acetate and –CO loss (MassBank Record: AU116102)).

It is especially challenging to elucidate the structure of TPs with a mass higher than *m/z* 326 due to the fact with an increase in the mass of fragments, the number of possible structures proportionally increases.

However, the presence of fragments that correspond in mass to other metabolites with a known structure may indicate that they may be the result of their conjugation or further modification and suggests metabolic pathways. So, metabolite DCF_M389 having characteristic fragment at *m/z* 294 corresponding to DCF_M294A and *m/z* 265 corresponding to -CO loss suggests that DCF_M389 can be a product of DCF_M294A modified by -CH_3 and -HSO_3 groups. Similarly, metabolite DCF_M374 has fragments characteristic for DCF_M294B (*m/z* 294, 276, 248) and may be its derivative containing a -CH_4SO_2 fragment.

The presence of a large number of fragments containing sulfur in DCF_M358 (down to *m/z* 262 corresponding to $C_{13}H_9ClNOS$) suggests that the sulfur atom can be attached to the nitrogen or directly to the aromatic ring of DCF. Similar conclusions can be drawn for DCF_M352 in which the sulfur atom remains in the smallest fragment at *m/z* 237 corresponding to the formula $C_{14}H_9N_2S$ (error 0.8 ppm). For the reasons described above, a similar position of the sulfur atom can also be found in the metabolites DCF_M343 and DCF_M329.

Important ions for the identification of conjugates could be fragments of DCF at *m/z* 278, 250 and 215, which corresponds to the loss of H_2O, CO_2 and -Cl. The presence of these fragments in DCF_M474 may suggest that these products were probably conjugates of 4′-hydroxy-diclofenac with the characteristic neutral loss of 164.1198 Da, which corresponds to the loss of $C_6H_{12}O_5$ (deoxyglucose), from the other hand DCF_M471 does not generate such fragments but its formula $C_{20}H_{19}Cl_2NO_8$ (error 0.4 ppm) suggests conjugation with $C_6H_8O_5$. Also specific can be fragments *m/z* 294, 275, 266 and 248 which correspond to the precursor ion of DCF_M294A/B and its fragments. These fragments were found in the products ions for substances DCF_M374 and DCF_M389 and corresponds to modification of DCF_M294 by -CH_2SO and -CH_3SO_3 groups respectively.

3.4. The kinetics of DCF metabolites formation

The abundance of DCF TPs was also studied at various timepoints of the experiment. A semi-quantitative approach based on peak areas was used to illustrate time profiles of different products. The levels of DCF_M278A, DCF_M278B, DCF_M286, DCF_M310.04, DCF_M312A, DCF_M312B, DCF_M326 and DCF_M358 in the culture medium constantly increased during exposure to DCF. The level of DCF_M250 increased up to 72 h and then remained unchanged, while DCF_M264, DCF_M294B, and DCF_M310.004 peaked at 24 h and then decreased gradually till the end of experiment (S6 Fig). A decrease in abundance over the course of the experiment could suggest that they are further degraded/transformed. The levels of all metabolites gradually increased throughout the experiment in the cell biomass (S7 Fig). The simultaneous increase in these TPs in biomass may be associated with a weakening of their release from cells, or can be explained by reabsorption into cells as a result of a weakened cell membrane semi-permeability caused by cell aging and the toxic effect of DCF [67,68].

Overall contribution of transformation products in the biomass as well as in the culture medium was calculated as a ratio of the sum of their peak areas vs. total area of all peaks (including DCF) on the chromatograms. TPs accounted for 18% in the biomass after 24 h exposure, reaching 31% by the end of the experiment (S8 Fig). In the nutrient medium, these indicators were 7% and 22.3%, respectively (S8 Fig).

4. Discussion

4.1. Phytotoxicity of DCF

While we observed a decreasing trend in the growth rate of all cultures from 24 h to 96 h, being the effect of general culture aging during prolonged batch culturing [69], in algae exposed to DCF the above-mentioned effect was more pronounced (S2 Fig). The adverse DCF effects were seen as a trend from the beginning of the experiment, however, statistically significant differences appeared after longer DCF treatment (48–96 h). A delay in DCF action could be the result of a relatively low concentration of the toxicant used in this study, i.e. 32.7 mg/L that was previously estimated as EC10/24 on the basis of the growth inhibition curve for *C. reinhardtii* cultures [27]. It is known [70] that some pharmaceuticals, that were classified as no-risk based on the acute toxicity tests (a classification of The Commission of the European Communities [71] related to chemical toxicities), when used at low concentrations can exert long-term effects. One of the examples is carbamazepine, for which the risk quotient (RQ) calculated from chronic assays was 60 times greater than acute RQ, because of potential bioaccumulation abilities [72]. In case of DCF, it seems that gradual DCF accumulation produces progressive disruption of cells functioning, since decrease in the population growth rate under DCF treatment was associated with photosynthesis disruption, visible from 48th h of exposure (S5 Fig). Further, after 72 h of DCF treatment, oxidative stress manifested in enhanced level of singlet oxygen could be seen in DCF-treated cells (S3Fig). This observation is in agreement with the phytotoxic effects of DCF described in our earlier publications on *C. reinhardtii* [27,29,30] as well as in the works by other authors reporting both photosynthetic processes inhibition and oxidative stress symptoms in DCF-treated

plants [24,26,73]. Even considering that experimental conditions (temperature, light intensity, nutrients supply etc.) can influence plants' sensitivity to pharmaceuticals, some DCF effects seems to be "universal" and phytotoxicity of this pharmaceutical is difficult to underestimate. The expected consequence of photosynthesis inhibition and oxidative-stress-related damage of cell components is dysregulation of metabolic processes. This seems to be supported by the observation that in cultures treated with DCF for 96 h, neutral lipids content was very limited as compared to control (S4 Fig). The accumulation of neutral lipids in 96-h control cultures is regarded as a natural process related to nutrients limitations and re-shuffling of carbon from carbohydrates to lipids [74] since numerous stress factors elicit the formation of lipid droplets in green algae [74–76]. In our experiment neutral lipids storage in DCF-treated cultures was significantly lower as compared to controls, indicating that lipid metabolism was affected by the drug. Whether the decrease in lipids synthesis and storage was the direct effect of photosynthesis inhibition, thus organic carbon shortage needs to be elucidated.

Since the main goal of our work was to estimate the algal potential for DCF removal, including its biotransformation, we paid special attention to the algae vitality during the experimental timespan. A fast and reliable indication of changes in the efficiency of photosynthesis, the process crucial for algae cells bioenergetics, is chlorophyll *a* fluorescence *in vivo*. Monitoring of quantum yield of photosynthesis (QY) allowed us to assume that in control conditions algae vitality remained at a quite high and stable level to the end of the experiment. Moreover, QY was only slightly reduced in DCF-treated cultures suggesting that the algae kept their metabolic activity at a relatively high level during the whole experiment, despite symptoms of oxidative stress (singlet oxygen overproduction) being observed in these cells. Thus, it can be assumed that DCF removal from the growth media can partially result from biotransformation processes, as discussed below.

4.2. Diclofenac removal

The ability of microalgae and higher plants to remove DCF from wastewater has been described [16,19,77–79]. Zhou et al. [15] have shown that *C. reinhardtii* can remove DCF from wastewater with high efficiency but the mechanisms of this process have not been studied. While higher plants are involved in the processes of biotransformation of DCF [24], Hifney et al. showed that the removal of DCF in *Chlorella sp.* is associated with biosorption processes [80], although the processes of enzymatic biotransformation of drugs are also described for *Chlorella* and some other microalgae [19,81–83]. Which of the above-mentioned processes (adsorption, absorption, or biotransformation) will prevail, depends not only on the organism but also on the experimental conditions and physicochemical properties of xenobiotics. For example, metals are actively biosorbed and bioaccumulated in *C. reinhardtii* [84–88]. For non-metals like selenium, *C. reinhardtii* has been shown to be capable of bioaccumulation and methylation [89,90], while complex organic compounds such as pesticides, phenol, and some other chemicals undergo both bioaccumulation and biotransformation [91–95].

While the concentration of DCF remained unchanged in abiotic controls (HSM A-F) during the experiment, DCF removal of about 37.7% was observed in *C. reinhardtii* culture. The adsorption by the cell wall, bioaccumulation inside the cells, and enzymatic biotransformation might be responsible for this decline, locating *C. reinhardtii* among the other algal strains that are effective in DCF removal from contaminated water, and for which literature data report the drug removal rates to range from 22% to 79% [10]. However, in our investigation relatively high initial DCF concentration was used, making *C. reinhardtii* a promising tool for remediation of highly contaminated environments.

To test the adsorption of DCF by the cell walls, its total content in the HSM collected after the pellet washing was determined. In terms of the total content of DCF in media, sorption by the cell wall was about 3%, of which only 0.04% was in the HSM after the second wash (leaching of intracellular DCF is negligible). Rinsing the biomass with a fresh medium makes it possible to estimate the amount of DCF loosely bound to the cell wall. This step takes into account only the physical interaction between DCF and the cell wall components and can be compared to models in which the sorption capacity is determined on the basis of adsorption isotherms calculated for dead biomass [96,97].

The sorption of DCF on the glass can be indirectly assessed using the HSM E and HSM D controls, in which, due to the absence of illumination, no degradation products were found and the decrease in the level of DCF (by 0.54%) can be explained only by sorption on glass. However, in the presence of living culture, sorption can be more intense due to the lower pH and the interaction of DCF with polysaccharides produced and excreted by algae. To assess the adsorption of DCF on the walls of glassware used for algae cultivation, we set an additional experiment: algae were exposed to DCF at a concentration of 9.19 mg/L (corresponds to EC1 to increase the duration of the experiment) for 20 days. On 10th, 15th and 20th day, the flasks in which they were grown were thoroughly washed with methanol (50 mL) to wash off the DCF adsorbed on the walls of the flasks. Subsequent quantitative analysis showed that, in terms of the total amount of DCF in the medium, only 0.06% was adsorbed on the flasks walls.

The accumulation of DCF in the biomass of *C. reinhardtii* was observed throughout the experiment and reached its maximum after 96 h. However, the calculation of the total content of DCF in the collected biomass showed that it accounts only for 2.4% of the total DCF amount in the culture, which is 13% of the total decrease in the drug concentration in the growth medium. It should be noted that, despite the small contribution of cellular uptake to the total DCF removal, the ratio between % of DCF accumulated by biomass and % of biotransformed DCF increased throughout the experiment (S9 Fig). It can be assumed, that a decrease in the rate of biotransformation would be a result of population aging and/or prolonged cells' exposure to the stress factor (DCF) leading to a slowdown in metabolism. In such conditions DCF accumulation into a biomass would prevail over the biotransformation. The accumulation of DCF over time may be also linked to a decrease in the pH of the growth media observed during the experiment. In an acidic environment, DCF dissociation (as a weak acid) can be reversed, promoting its sorption to biomass and contributing to its buildup over time.

Such a low content of DCF in the algal biomass can be explained by a large contribution of biotransformation processes in the total DCF removal. Also, the discrepancy between the decrease in the concentration of DCF in the growth medium and its content in the biomass can be explained by the fact that DCF and its metabolites are immobilized due to the formation of a non-extractable residue, probably due to the incorporation of conjugates into cell walls and other cell compartments. The formation of a non-extractable residue is usually considered as an additional detoxification mechanism (Phase III metabolism) [62].

Summarizing all of the above, the overall mass balance of DCF at the end of the experiment is as follows: 62.3% remained in the media; 8.41% was converted into transformation products (based on a semi-quantitative approach comparing the sum of transformation products peak areas with the peak area of DCF); 2.4% was absorbed in the biomass (into the cells); 2.96%, adsorbed on the cell wall; 1.4% the loss is associated with photodegradation, and 0.54%, with adsorption on the walls of the culture vessels; the fate of the remaining 22% is unknown (Fig. 2). Possibly, as mentioned above, DCF or its transformation products are bound to the cell wall or the other subcellular structures and are in a non-extractable form [62]. It is also not excluded that DCF underwent a further transformation to low-molecular-mass compounds we were unable to identify if DCF was removed due to the formation of volatile derivatives (see subchapter "Diclofenac biodegradation"). At the same time, it should be remembered that the level of DCF TPs was quantified by their peak areas and, when the TPs lose their functional groups, this can largely affect the effectiveness of ionization [98]. Also, as mentioned above, the extraction, identification method and MS conditions were optimized for DCF, therefore, the signal intensity and

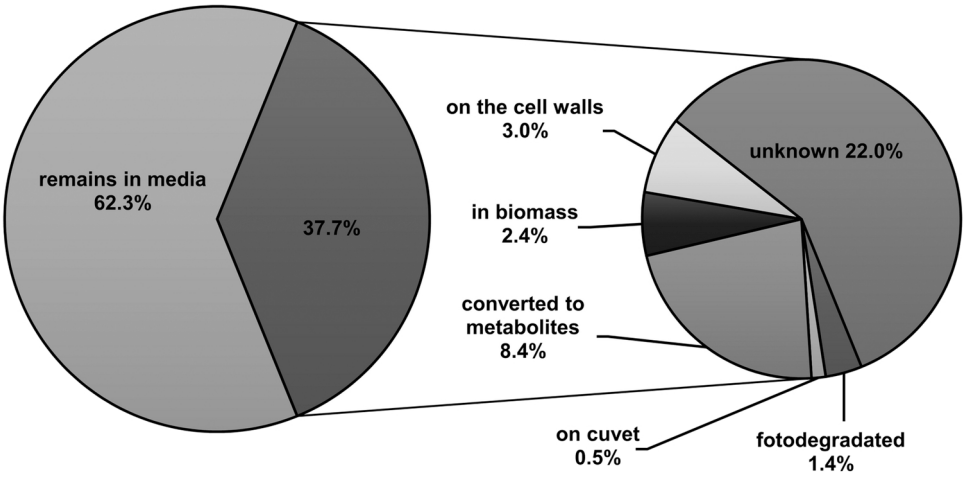

Fig. 2. The fate of diclofenac 4 days after adding it to a nutrient medium with *C. reinhardtii*.

the peak area for TPs may be underestimated (possibly, some TPs cannot be detected with the same analysis mode) [41]. Additional studies with radiolabeled DCF may provide an answer to this question.

Concluding, the mass balance study indicates that DCF removal cannot be fully explained by the processes of sorption and biotransformation. We hypothesize disappearance of a significant part of DCF by its binding to a non-extractable residue (phase III) or complete decomposition in metabolic cycles of *C. reinhardtii*. Even though biotransformation seems to be promising mechanism of pharmaceuticals removal from the aquatic environment, the risk of formation of highly toxic intermediates should be also considered.

4.3. Diclofenac biodegradation

The experiments allowed to identify several products of the transformation of DCF, and despite the fact that the structure of some TPs found by us was not unambiguously determined, it is possible to suggest metabolic pathways for the transformation of DCF based on the literature data on the biotransformation of DCF in other organisms (higher plants, bacteria, fungi) as well as based on information about the specific mechanisms of biotransformation of other xenobiotics in *C. reinhardtii*.

The biotransformation pathways of DCF in humans and animals are well described [63,99], while little is known about the metabolism of DCF in plants and especially in unicellular algae. The main and common pathway for biotransformation of DCF for most living organisms is oxidation using cytochrome P450 (CYP450) systems which is the I phase of metabolism followed by a II phase resulting in the formation of conjugates [8]. In plants and animals, both phases of DCF metabolism occur [100], and during the second phase, conjugates with glucuronic acid, sulfate, amino acids, glucose and glutathione are formed [62]. In addition, recent studies show that glutathione S-transferase and laccase may play a decisive role in the detoxification of many xenobiotics in *C. reinhardtii* [94].

The main metabolic pathways of DCF in *C. reinhardtii* can be indirectly suggested based on the levels and time profiles of individual DCF TPs.

In quantitative terms, the most pronounced metabolites found in the biomass are DCF_M250, DCF_M310.04 and DCF_M278A, the level of which increases with exposure time. The structure of those metabolites shows that they are formed in the processes of decarboxylation, amidation and methylation, respectively. In the growth medium, the levels of DCF_M250 and DCF_M278 were also higher than other metabolites but the highest level reached the DCF_M312A, which can be explained by its rapid release from the cell due to its greater hydrophilicity. Unlike other studies [62] in which an increase in the DCF_M312A level was observed at the beginning of the exposure and then decreased due to its further transformation (for example, conjugation), in our study, the DCF_M312A level constantly increased during the experiment. However, a decrease in levels during exposure was observed for DCF_M294B, which is formed as a result of amidation of DCF_M312B and for DCF_M264 (dehydroxylation of DCF), which may indicate activation of further conversion of these compounds (Fig. 3), the weakening of their production or less output from the cells, since the levels of these metabolites decreased only in the nutrient medium, while their accumulation took place in the biomass.

The methylation of the DCF catalyzed by carboxylic acid O-methyltransferases, which we observed is also noted during biotransformation of DCF by some bacterial strains [65] and by animal aquatic organisms [101]. While O-methylation is rare in higher plants, a similar pathway of xenobiotic biotransformation in phytoplankton has been described [17]. *C. reinhardtii* has a broad spectrum of methyltransferases and uses methylation processes to detoxify selenium [49]. In other studies, hydroxylated DCF derivatives are methylated to form DCF_M326 [65, 101–103] and DCF_M340 [101,104] by bacteria and fungi, but in our study, DCF was methylated directly to form DCF_M310.04 similarly to animals [101].

Hydroxylation of one of the two phenyl rings of DCF, which is catalyzed by P450 monooxygenases in most organisms, is considered the first phase of xenobiotics metabolism and leads to the formation of − 3, − 4, − 5, and − 4/− 5 hydroxyl products [5,8,9]. In algae, hydroxylation catalyzed by CYP450 monooxygenase is also the main mechanism of the first phase of biotransformation [81]. Nazos et al. showed that biodegradation of phenol by *C. reinhardtii* is an oxygen-dependent process [105]. Wang et al. showed that *C. reinhardtii* uses CYP450 in biotransformation of triazophos [92]. Therefore, it was expected that 4-hydroxy-diclofenacwould be one of the main metabolites in *C. reinhardtii*.

Despite the fact that CYP450 can induce biological reactions of reductive dehalogenation [50], and dechlorinated DCF metabolites were found in bacteria [102,106,107], and for *C. reinhardtii* during biotransformation of triclosan [94], we did not find respective metabolites of DCF in this study.

The acetylation reactions belong to the II phase of metabolism and are characterized by the transfer of the acetyl moiety and the donor is usually the acetyl-coenzyme A. The formation of aceclofenac can occur in the case of O-acetylation of DCF, although in the environment aceclofenac can be metabolized with the formation of DCF as an intermediate [108]. It is known that plants, bacteria, and fungi use O-acetylation to modify their cell walls [109,110], possibly *C. reinhardtii* can use this pathway for biotransformation of xenobiotics, especially since the use of O-acetylation for xenobiotic elimination has also been shown for *Plantago lanceolata* [111], *Nicotiana tabacum* [68], *Medicago sativa* [112] and

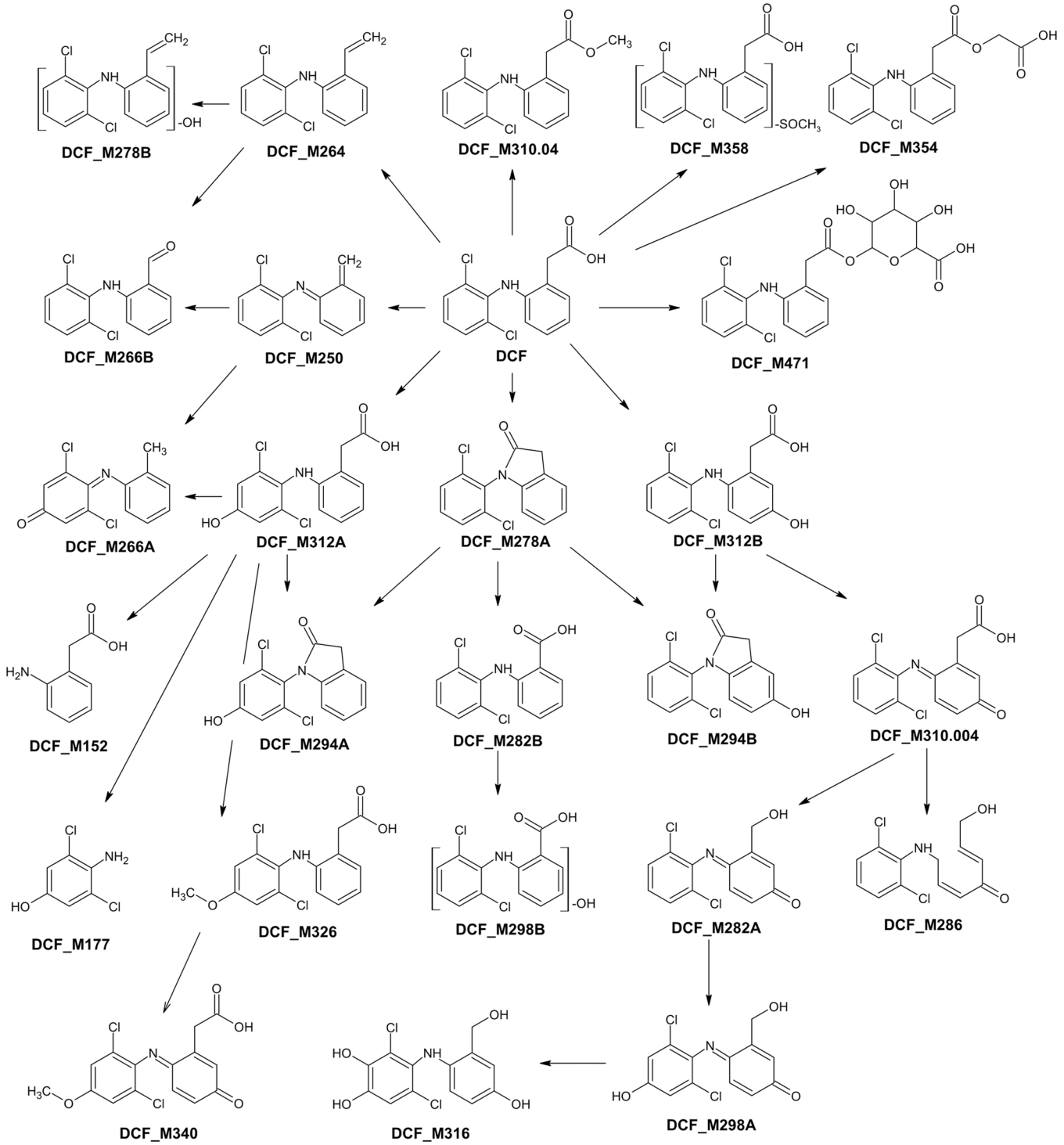

Fig. 3. Proposed DCF degradation intermediates produced by *C. reinhardtii*.

for *Campanula rotundifolia* [113], although there is no information on enzymes that catalyze O-acetylation of drugs in plants [114].

Along with the most quantitatively expressed metabolites (DCF_M278A, DCF_M310.04B, DCF_M312A, DCF_MB and DCF_M358), the formation of the DCF_M250 metabolite makes a large, quantitative contribution, especially among biomass metabolites. Based on the proposed structure for DCF_M250, it can be assumed that it is formed as a result of decarboxylation of DCF. Despite the fact that a similar transformation pathway of DCF is often characteristic to abiotic transformation (S1 Table), experiments carried out to study the biological transformation of DCF using various modifications of activated sludge showed that decarboxylation reactions take a great place [102]. In addition, these reactions are often associated with subsequent oxidation reactions, which can explain the appearance of metabolites DCF_M266A and DCF_M266B.

The high content of DCF_M310.04 in biomass can be explained by its high hydrophobicity compared to other metabolites, for this reason methylated DCF has the highest bioconcentration factor (BCF) values and thus can be toxic for non-target species [101].

Another pronounced metabolite DCF_M278 (DCF-lactam) formed

during the dehydration of DCF is widely found during biotransformation by microbes and plants (S1 Table). Opening of the lactam ring with subsequent oxidation or decarboxylation reactions can lead to the formation of metabolites DCF_M282B, DCF_M294A and DCF_M294B [115].

The formation of DCF-glucuronide is well known in animals [57,63,101,116] and higher plants [24,62,100]. Phytoplankton is known to use the processes of conjugation of xenobiotics (including pharmaceuticals) with glucose [17,117]. Wang et al. demonstrated the possibility of the formation of glycosylated metabolites of triclosan in *C. reinhardtii* [94], therefore, the detection of DCF_M471 - the conjugate of DCF with glucose was expected. It should be noted that the binding of glucose to the carboxyl group of DCF, which is observed in our experiments, is more typical in animals [57,63,101,116], while in plants, conjugation by binding to a hydroxyl group has been described [24,62,100]. But this is not so controversial considering that in *C. reinhardtii*, conjugation of glutamate with mefenamic acid (structurally similar to DCF) also goes by binding to the carboxyl group [17]. Similarly, diatom *Navicula sp.* is able to bind glucose to the carboxyl group of ibuprofen [117]. The formation of conjugates with glucose can be important detoxification of DCF since these products can later be incorporated into the lignin fraction of the cell wall, thereby becoming non-extractable residues [118].

The formation of DCF conjugates with amino acids in the course of metabolism is specific to animals [119]. Despite the fact that Fu et al. showed that conjugates with amino acids are one of the main metabolites of DCF in higher plants [62], in our study such conjugates were not found.

In our work, 6 metabolites containing sulfur were found, but unfortunately, insufficient fragmentation data and the absence of MS spectra in the literature did not allow us to identify the structure of these metabolites and assure that they are the result of sulfate conjugation of the DCF. Perhaps, in our case, sulfhydrylation occurs, which is also an important biotransformation pathway in *C. reinhardtii,* for example, when exposed to triclosan [94]. Also, in the process of DCF biotransformation, sulfurated metabolites such as thiol-, cysteine (Cys) - and GSH-conjugates can be formed, which are more hydrophilic and have lower toxicity [94].

If we compare the metabolites we found with the biotransformation products of DCF described in the literature (S1 Table), it can be noted that metabolites with a relatively low molecular weight (DCF_M152, DCF_M177, DCF_M250, DCF_M266, DCF_M278, DCF_M282, and DCF_M298) are formed by bacteria, fungi and under the influence of abiotic factors. Metabolite DCF_M294 and others with higher molecular weights have been found in fungi, plants, and animals. On the one hand, this may indicate a great similarity of the metabolic pathways of *C. reinhardtii* with bacteria, on the other hand, metabolite DCF_M471 is characteristic only to higher plants and animals and does not occur in and is not typical for bacteria [120].

An important role in the biodegradation of DCF is played by laccase enzymes, which can neutralize both DCF itself and its potentially hazardous products by opening and hydroxylation the ring and final mineralization to CO_2, NH_3, H_2O, and chlorine. At the moment, there are reports of the use of laccase synthesized by fungi for these purposes [121]. *Chlamydomonas moewusii* possesses natural laccases capable of neutralizing phenolic compounds [122]; in addition, genetic modification of *C. reinhardtii* is carried out with the use of fungi laccases to improve the destruction of micro-pollutants (including pharmaceuticals) [123]. Given that genes encoding laccase have been detected in *C. reinhardtii* [94,124], DCF can form a para-hydroxy-substituted intermediate, which can undergo further oxidation to form a para-benzoquinone-imine derivative (DCF_M310.004) as a result of a laccase-mediated oxidation reaction. Also, the laccase mechanism may underlie the formation of metabolites DCF_M152 and DCF_M177 as a result of C-N cleavage [121].

The scheme of DCF metabolic pathways proposed here (Fig. 3) is consistent with the fact that metabolites whose levels decreased (DCF_M264, DCF_M294B, DCF_M310.004) in the nutrient medium probably are intermediates, and those whose levels linearly increased (DCF_M278B, DCF_M286, DCF_M310.04, DCF_M358) are final metabolites, except DCF_M278A, DCF_M312A/B, DCF_M326 and DCF_M250 which, according to the scheme, are precursors. This may be explained by the fact that proposed end-products appeared only on the last day of the experiment in very small amounts (maybe due to their abiotic origin) and did not affect the level of other metabolites. Although our proposed pathway of DCF conversion to DCF_M316A coincides with that described for bacteria, in which the authors associate the formation of metabolites DCF_M298A and DCF_M316 with hydroxylation by a monooxygenase [65]. Thus, we have found a wide range of DCF transformation products in *C. reinhardtii* suggesting metabolic pathways similar to those of animals, bacteria, and higher plants. The results lead to better understanding of the metabolic fate of DCF in freshwater algae *C. reinhardtii* and thus may provide insight into the degrading/detoxifying potential of these algae for other NSAIDs with a similar structure.

5. Conclusions

Our work has shown that *C. reinhardtii* can to some extent remove DCF from the water environment. Based on the analysis of biological parameters, it was shown that, even with a high concentration of DCF, *C. reinhardtii* keeps its vital parameters at a high level. A wide range of I and II phase DCF metabolites was found, wherein in *C. reinhardtii*, many metabolic pathways are more similar to those in animals and bacteria than in higher plants. Among 38 detected DCF transformation products, 18 were reported for the first time. Therefore, our results enabled us to propose a complex scheme of DCF decomposition pathway in the presence of *C. reinhardtii* in the aquatic environment, which provides new insights into bioremediation potential of green algae and can contribute significantly to broadening the knowledge about the xenobiotic transformation by these organisms.

Our research opens new perspectives in the field of phycoremediation. First, the reasonable direction for further research seems to be evaluation of toxicity of TPs formed as a result of algal metabolism. Secondly, remediation potential of *C. reinhardtii* towards other NSAIDs should be explored. And finally, detail information about the main pathways of NSAIDs metabolism is needed to enhance the efficiency of key cellular systems involved in biotransformation, which could contribute to the development of bioremediation techniques based on the use of microalgae.

Environmetal implication

Our study aimed to evaluate the potential of the green alga Chlamydomonas reinhardtii to remove non-steroidal anti-inflammatory drugs (NSAIDs), classified as contaminants of emerging concern, from the aquatic environment. NSAIDs are continuously introduced to water bodies causing a significant threat to non-target organisms, thus remediation of NSAIDs-contaminated water is a matter of high priority. Diclofenac, widely detected in various types of environmental waters, was chosen as a model compound. We have found that C. reinhardtii reduces diclofenac level in the growth media via both sorption and biotransformation processes, thus, our research opens new perspectives in the field of phycoremediation.

CRediT authorship contribution statement

Ivan Liakh: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Darya Harshkova:** Formal analysis, Investigation, Writing – original draft. **Pavel Hrouzek:** Conceptualization, Supervision, Writing – review & editing. **Kateřina Bišová:** Investigation, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Anna Aksmann**: Conceptualization, Project administration, Writing – review & editing,

Funding acquisition, Writing – original draft. **Bartosz Wielgomas:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131570.

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