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Removal of Estradiol, Diclofenac, and Triclosan by Naturally Occurring Microalgal Consortium Obtained from Wastewater

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Abstract: The occurrence of emerging contaminants like pharmaceutical drugs and personal care products in aquatic systems is now being identified as a potential risk to human health. Since conventional wastewater treatment systems are unable to remove them sufficiently, high concentrations of some of the commonly used drugs are reported to be occurring in many effluents. Microalgae-based systems have been investigated in recent years as an environmentally safe alternative to chemical oxidation methods for elimination of these emerging contaminants. Therefore, a process utilizing the microalgal consortium was assessed for its potential to tolerate environmentally high concentrations of pharmaceutical drugs and also to simultaneously remove the synthetic hormone estradiol, anti-inflammatory drug diclofenac, and antibacterial agent triclosan. The effective concentration with 50% mortality for the consortium was determined for each contaminant as 16, 8, and 8 mg L^{-1} for estradiol, diclofenac, and triclosan, respectively. These three drugs were then spiked separately in algal growth media at effective concentration with 50% mortality, and the microalgal growth in presence of these drugs and the drug removal was monitored in shake-flask setup. The study shows substantial removal of estradiol 91.73% ± 0.0175, diclofenac 74.68% ± 0.0092, and triclosan 78.47% ± 0.015 by the microalgal consortium during their growth phase. Further, it was observed that degradation of the drugs by microalgae was the prominent removal mechanism and not adsorption. Estradiol and diclofenac did not show any immediate negative impacts on the microalgal growth as seen from the biomass and chlorophyll content measurements. However, triclosan proved detrimental to the microalgal growth as the consortium did not survive beyond 5 days after spiking. Promising results for emerging contaminants removal was obtained, and a treatment system can be designed to remove different drugs from wastewater by using the naturally occurring microalgal consortium.

Keywords: emerging contaminants; microalgal remediation; pharmaceutical drugs

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1. Introduction

Water contamination by the conventional class of pollutants like phosphate, nitrates, etc. has been extensively studied. However, in recent years, the contamination of water bodies with newly synthesized anthropogenic chemicals has emerged as a major issue of concern. A new class of contaminants, called emerging contaminants (ECs), are now being recognized as a major potential threat to human health and the environment. These include pharmaceutical and personal care products (PPCPs), industrial chemicals, solvents, pesticides, volatile compounds, and other newly discovered compounds [1]. These varieties of compounds have complex functions and mechanisms of action. However, their effects on the non-target organisms in the aquatic environment like invertebrates and fishes are unknown [2]. After their release into the environment, some of these compounds may still have the potential to exert negative impacts by synergistic/or additive effects when occurring together in a mixture in aquatic medium [3]. Additionally, the long-term effects on the whole ecosystem due to continuous exposure to low doses of these compounds is

not well studied. It has been found that some of these ECs can pose a significant threat to aquatic organisms even at very low concentrations [4,5]. The occurrence of antibiotic drugs in water bodies causes antibiotic resistance in microorganisms, leading to the emergence of multidrug-resistant infections in human beings [6].

With advancements in technology and new drug discoveries in recent years, the worldwide usage of these newly discovered pharmaceuticals has been continuously increasing. Several studies have also reported the concentrations of some of these ECs even exceeding 1 mg L^{-1} in treated industrial effluents and recipient waters. Hospitals are considered as major sources of pharmaceutical compounds in the environment [7]. The effluents from hospitals contain active principles of drugs and their metabolites, other chemicals, heavy metals, disinfectants, etc. [8]. High concentrations of many commonly used pharmaceutical drugs like furosemide (0.018 mg L^{-1}), salicylic acid (0.07 mg L^{-1}), erythromycin (0.027 mg L^{-1}), ciprofloxacin (0.124 mg L^{-1}), diclofenac (0.07 mg L^{-1}), ibuprofen (0.151 mg L^{-1}), acetaminophen (1.369 mg L^{-1}), iobitridol (3.213 mg L^{-1}), etc. have been reported in hospital effluents [9].

However, hospitals cannot be considered as the only source of pharmaceuticals in the environment. Conventional wastewater treatment systems were designed to remove pollutants like organic carbon, phosphate, nitrates, etc. They are neither designed nor capable of removing these ECs that are released from other domestic, industrial, and agricultural sources. Therefore, the pharmaceuticals escape these treatment systems and enter the environment in an untreated or partially treated form [10]. Residues of pharmaceuticals have widely been reported in wastewater treatment plant (WWTP) effluents in concentrations ranging from 0.001–55 μg L^{-1} with removal efficiencies varying from 12.5% to 100% for different ECs [11,12]. A few studies have been done specifically in India around pharmaceutical manufacturers and have found extremely high levels of many commonly used drugs. Larsson et al. 2007 [13] analyzed the effluent from a drug manufacturing unit in India and reported high concentrations of ciprofloxacin at 28–31 mg L^{-1} and fluoroquinolones at 0.15–0.9 mg L^{-1}. High ciprofloxacin concentrations have been reported even in sediments present downstream of an industrial WWTP in India [14]. Another study in Hyderabad, India by Lubbert et al. 2017 [15] near a drug manufacturing site reported high concentrations of moxifloxacin (0.69 mg L^{-1}], voriconazole (2.5 mg L^{-1}), and fluconazole (240 mg L^{-1}). The effluent from a pharmaceutical manufacturing facility in China reported a concentration of 20 mg L^{-1} of oxytetracycline in treated effluent, signifying that these compounds easily escape the treatment system [16]. A study in Pakistan found 27 mg L^{-1} of oxytetracycline, 28 mg L^{-1} of trimethoprim, and 49 mg L^{-1} of sulfamethoxazole, as well as many other antimicrobials downstream of drug-formulation facilities [17]. In China, the concentration of synthetic hormone ethinylestradiol was found to be 51 ng L^{-1} even in a treated effluent [18]. Elevated levels of pharmaceutical drugs are expected to occur in high population-density areas, due to extensive use of the common over-the-counter drugs. Consequently, many studies have reported elevated concentrations of many drugs in effluents in India and China. However, there are many related reports from antimicrobial manufacturing facilities all around the world [13]. Even in a developed country like Korea, concentrations of up to 44 mg L^{-1} of lincomycin were found in effluent from a pharmaceutical manufacturer [19]. In Croatia, concentrations up to 3.8 mg L^{-1} of azithromycin were found in effluent from a pharmaceutical WWTP.

Microalgae-based treatment systems are widely investigated as an alternative for wastewater treatment as the conventional wastes like carbon, nitrogen, and phosphates constitute a nutrient source for them [20]. This process is solar energy driven and eco-friendly, and also has a role in carbon-dioxide fixation and the production of some high-value products. In addition to the removal of conventional contaminants, some recent studies have also demonstrated that the microalgae performed compound-, species-, and environment-dependent removal efficiencies of different ECs in wastewater [21]. It has been reported that the presence of contaminants in the external environment of microalgae can produce oxidative stress in them, triggering an antioxidant response, which further regulates the

algal growth processes [22–24]. Additionally, antioxidant responses in microalgae have been proven to be related to the degradation of exogenous chemicals such as glutathione and glutathione S-transferase [25]. Therefore, the present study was taken up to assess the removal of three selected commonly used drugs using a microalgal–bacterial consortium under controlled greenhouse conditions.

The three selected compounds for the study are described as follows. Estradiol (also known as 17β-estradiol, EST) is a natural hormone in the human body. It is the major female sex hormone in humans and is the most effective mammalian estrogenic steroids; hence, it is widely used in several hormone therapies. Estradiol, estrone, and estriol are excreted in the urine along with glucuronide and sulfate conjugates. Diclofenac (2-(2-(2,6-dichlorophenylamino) phenyl) acetic acid, DCF) is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. It is a commonly used drug throughout the world in tablets, solutions, or gels and sold under many commercial names. After undergoing metabolic activities within the body, 60–70% of the dose is excreted in the urine and approximately 30% in the bile as conjugates of unchanged diclofenac plus metabolites [26]. Triclosan (TCS) is widely used as a preservative and antimicrobial agent in personal care products as well as in household items. In humans, the resulting conjugates are excreted primarily in urine [27]. Therefore, these three pharmaceutical drugs EST, DCF and TCS were selected for the present study to assess the removal of their high-spiked concentrations using the microalgal consortium obtained from a lake receiving untreated sewage in New Delhi, India.

2. Materials and Methods

2.1. Algal Consortium, Culture Medium, and Growth Conditions

The microalgal consortium used in this study was taken from the algal culture collection of Applied Microbiology Lab, Indian Institute of Technology Delhi. This microalgal consortium was earlier obtained from the naturally occurring algal bloom growing throughout the year in Hauz Khas Lake (South Delhi, India), which receives treated effluent from the Vasant Kunj sewage treatment plant and untreated wastewaters from other drains. It has been reported that the consortium has more than 6 types of microalgal cells and some bacterial strains, with *Chlorella* sp., *Merismopedia* sp., *Closteriopsis* sp., and *Scenedesmus* sp. as the dominant species [28]. The inoculum for the experiment was cultivated in 1000 mL Erlenmeyer flasks in 700 mL BG-11 culture medium in an algal cultivation chamber under controlled greenhouse conditions of temperature (25 ± 1 °C) and irradiance (5 ± 0.5 Klux) using florescent white light, with 12:12 h light-dark cycle.

2.2. Chemicals and Reagents

For the preparation of standard and spiking solutions for the experiment, pharmaceutical secondary standards of EST (ß-Estradiol, CAS number 50-28-2), DCF (CAS number 15307-79-6, as sodium salt), and TCS (CAS number 3380-34-5) were purchased from Sigma–Aldrich (Steinheim, Germany). The chosen drugs are widely used, persistent, and not readily degradable in the conventional municipal wastewater treatment plants. Methanol, acetonitrile, and water (HPLC grade) were purchased from Merck (USA). Analytical-grade water was obtained from RIONS Ultra 370 Series Milli-Q water purification system. BG-11 algal growth media were procured from HiMedia (India). Then, 100 mg L^{-1} of stock solution and the spiking solutions of each drug were prepared in HPLC-grade methanol.

2.3. Experimental Setup

To carry out this study, shake-flask experiments were set up in the greenhouse. The microalgal consortium, grown under the conditions mentioned in Section 2.1, was used as inoculum when its optical density reached 2. About 30% of this exponentially growing inoculum was added in sterile algal growth media and spiked with each of the three drugs EST, DCF, and TCS separately. The flasks were shaken manually twice a day.

The flow of the experiments is shown in Figure 1 and described as follows. Firstly, the effective concentration with 50% growth inhibition (EC-50) was determined for each drug. This EC-50 concentration of each drug was then tested for removal by the microalgal consortium by two processes—adsorption and degradation. Secondly, an adsorption study for drug removal was carried out for 12 h. Samples were collected for HPLC analysis from both the culture media and the microalgal cells. Thirdly, a degradation study for drug removal was subsequently done. Negative and positive controls were also set up to assess the abiotic degradation of the drugs under the experimental conditions and microalgal growth in the absence of the drugs, respectively. Samples were collected every day to monitor microalgal growth (optical density at 680 nm and chlorophyll content) and the removal of the drug from the culture medium during the experimental period.

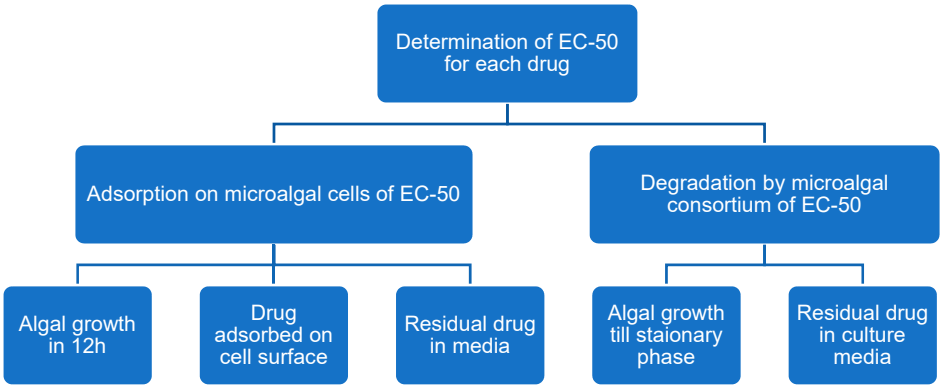


Figure 1. Flow of the experiment for the study of selected EC removal by microalgal consortium.

HPLC analysis was done to assess the drug adsorption on cell surface and residual concentration of the drugs in the culture medium. SEM analysis was also carried out for microalgae at the end of the degradation experiment to assess the changes in cell morphology due to the drug in the culture media. Details for each of these steps are described in detail in the following sections.

2.3.1. Growth Inhibitory Concentrations for EST, DCF, and TCS

The growth inhibitory concentration of each drug was assessed using the ecological effects test guidelines EPA 712-C-006 [29], which were modified for the microalgal consortium used in the current study. Briefly, the microalgal culture was exposed to different test concentrations of each of the drug in geometric ratio (0, 2, 4, 8, 16, 32, and 64 mg L^{-1}) in duplicates in 250 mL flasks. Optical density and microscopic observations were made every 24 h, until 72 h. The microalgal specific growth rate (μ) was calculated by Equation (1) as follows:

$$\mu = \{logn(Di) - logn\ (D0)\}/t \quad (1)$$

where: $D0$ = the cell concentration measured as optical density at 680 nm at the initial day; Di = the cell concentration measured as optical density at 680 nm on the day i; and t = time interval from 0 to i in days.

Percentage growth inhibition (%I) at each test concentration at 72 h was determined usin Equation (2):

$$\%I = \{(C - X) * 100\}/C \quad (2)$$

where:
C = the control specific growth rate;
X = the treatment specific growth rate.

The percentage growth inhibition of each drug was plotted at different test concentrations to obtain the half-maximal effective concentration (EC-50). This EC-50 concentration

was used as the spiking concentrations for the adsorption and degradation studies by the microalgal consortium.

2.3.2. Drug Adsorption on the Microalgal Surface

For the study of drug adsorption on cell surface, about 30% of microalgal inoculum was added to 350 mL sterilized growth media and spiked with freshly prepared drugs at the EC-50 concentration, as previously determined. Microalgal growth and drug adsorption on the microalgae was studied by sampling in the initial 12 h of the experiment and at every 2 h interval.

2.3.3. Drug Degradation by Microalgae

The drug degradation was tested for each drug spiked at EC-50 concentration in shake flasks at 300 mL volume in three sets: (a) the drug removal was tested by microalgae-inoculated culture medium with the spiked drug; (b) a positive control with only the microalgae-inoculated culture medium, but without drugs (Control microalgae), was assayed for microalgal growth in absence of any pharmaceutical drug; and (c) a corresponding negative control was also assayed with only the drug in the culture medium, and without microalgae (Control EST, Control DCF, and Control TCS) to test any abiotic degradation of the drug under the experimental conditions. Samples were collected every 24 h. The degradation experiment was carried on until the stationary phase of the microalgal growth was seen. Experiments were carried out in duplicate under identical conditions.

Throughout both the adsorption and degradation experiments, the growth of the culture was monitored measuring the optical density at 680 nm and chlorophyll estimation was done by taking an aliquot of 2 mL from each of the flasks for the analytical determinations. All measurements were done in triplicates. Samples were collected for microscopic analysis at the end of the degradation experiment to assess any morphological changes induced due in the microalgal cells to the drug spiking.

2.4. Analytical Methods

2.4.1. Algal Density and Chlorophyll Analysis

Optical density of the culture media was measured at 680 nm by PerkinElmer UV/VIS LAMBDA-35 spectrometer.

For chlorophyll estimation, 2 mL of culture broth was centrifuged at 10,000 rpm for 10 min, and then the pellets were treated with methanol and kept in a water bath for 30–40 min at 60 °C. Optical density was taken at 652, 665.2, and 750 nm by UV spectrophotometer [30]. Chlorophyll content was determined using Equation (3) as follows:

$$Chlorophyll\ a\ content\ \left(\mu\frac{g}{mL}\right) = \{16.29 * (A665.2 - A750)\} - \{8.54 * (A652 - A750)\} \quad (3)$$

where:
$A652$ = Optical density at 652 nm;
$A665.2$ = Optical density at 665.2 nm;
$A750$ = Optical density at 750 nm.

2.4.2. HPLC Analysis of the Drugs

Analysis of Drug Adsorbed on Microalgal Surface

The concentration of the spiked drug that may be present on the microalgal cells due to adsorption on the cell surface was analyzed using the HPLC method [31]. Briefly, the microalgal pellets obtained after centrifugation of the collected samples in the above step Section 2.4.1 were mixed with 5 mL acetonitrile and sonicated at 35 kHz in a Labman Ultrasonic cleaner (LMUC-3) for 20 min at room temperature. Then, 2 mL of the extract was evaporated until there was dryness in nitrogen stream. The precipitate was then dissolved in methanol and diluted with Milli Q water in ratio of 1:1 and injected into the column for analysis. The HPLC system consisted of an Agilent 1260 infinity series with

an Agilent Eclipse Plus C-18 column (5 µm, 4.6 × 150 mm). Eight-point calibration curves were prepared with standards in HPLC methanol.

The residual drug concentration in the culture media was also determined by HPLC. The residual EST in the culture medium in each sample was assessed using acetonitrile: water (6:4) as the mobile phase, at a flow rate of 1 mL per minute and a detection wavelength at 202 nm [32]. For DCF analysis, the mobile phase was composed of acetonitrile:water:orthophosphoric acid (70:30:0.1, $v/v/v$), with a flow rate of 1 mL min^{-1} and a detection wavelength at 276 nm [33]. For TCS analysis, the mobile phase used was methanol:water (90:10) at a flow rate of 1 mL per minute and a detection wavelength at 280 nm [34]. The mobile phases were degassed in an ultrasonic bath for 30 min prior to analysis. The samples were centrifuged and filtered through 0.22 µm glass-fiber filters before injection into the system.

Analysis of Residual Drug in Culture Media

The removal of the spiked drug by degradation was assessed by measuring the residual concentration of the drug in the culture media at different time intervals using HPLC, as described above.

2.4.3. Specific Removal of Drugs

For estimating the biomass, 30 mL microalgal samples were collected and centrifuged at 8000 rpm. After discarding the supernatant, the pellets were kept at 60 °C for 48 h in an oven for drying, until a constant weight was achieved. The total biomass production was calculated using Equation (4):

$$Biomass\ production = \frac{total\ dried\ biomass}{volume\ of\ media} \quad (4)$$

Specific removal of drugs by the microalgal consortium can be calculated using Equation (5):

$$Specific\ removal\left(\frac{mg}{g}\right) = \frac{total\ removal\ of\ the\ drug}{total\ biomass\ of\ microalgae} \quad (5)$$

2.4.4. Scanning Electron Microscopy of Microalgal Cells

Scanning electron microscopy (SEM) was done to determine any morphological changes in the microalgae growing in the drug-spiked media, due to any stress/toxicity induced by the pharmaceutical drug as compared to the control microalgal samples with no drug spiking. The process for SEM sample preparation is described briefly: 2 mL microalgal suspension samples were centrifuged at 9000 rpm for 15 min [35]. Supernatant media were removed and the resulting pellets were resuspended in 0.15 M phosphate buffer saline (PBS, pH 7.2). The remaining samples were then washed twice in PBS, followed by fixation in 100 µL 2.5% glutaraldehyde at 4 °C overnight. The pellets were then washed again with PBS thrice and stored at −20 °C for 24 h. The frozen samples were then lyophilized for 48 h. The dried biomass samples obtained after lyophilization were mounted on aluminum stubs for gold-coating by cathodic spraying (Polaron gold). Microscopic analysis was carried out using SEM ZEISS EVO 50.

2.5. Statistical Analysis

All the measurements and reading were taken in triplicates and the results are expressed in terms of mean ± standard deviation. For statistical analysis, XL-STAT 2016 was used. Both independent variable *t*-tests and a parametric one-way analysis of variance (ANOVA) were performed. Correlation analysis was performed between algal growth and drug removal. The statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1. Growth Inhibitory Concentrations for EST, DCF, and TCS

The microalgal consortium growth response to different concentrations of the spiked drug in the growth media was estimated by the specific growth rate and percentage growth inhibition for each concentration plotted for each drug (Figure 2). In the preset study, it was observed that the EC-50 for EST, DCF, and TCS was 16, 8, and 8 mg L^{-1}, respectively, as seen in Figure 2.

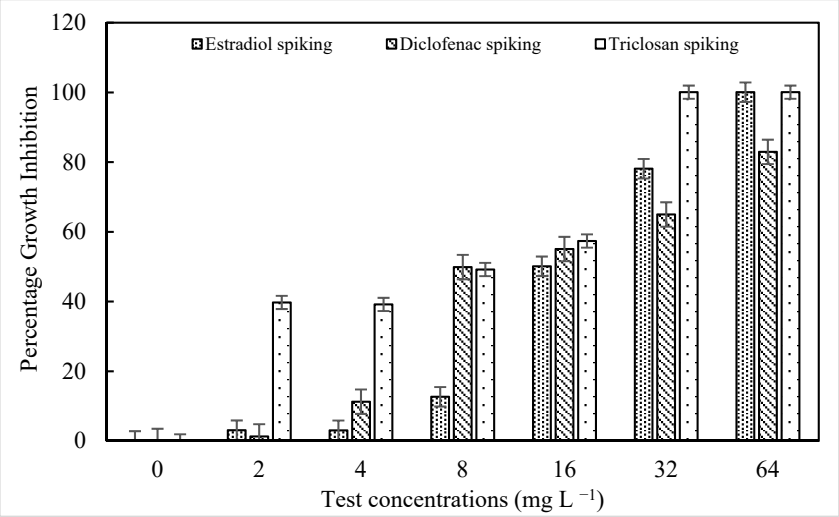


Figure 2. Percentage growth inhibition of microalgal consortium by estradiol, diclofenac, and triclosan spiking at different concentrations for 72 h.

It has been reported in other studies that microalgae are influenced by the ECs present in their environment. However, the microalgal sensitivity and tolerance towards these compounds has been observed to be species dependent and related to the specific contaminant. Therefore, different classes of compounds show different levels of toxicity for microalgae. *Raphidocelis subcapitata* was reported to be tolerant to synthetic hormone 17-β estradiol at concentrations up to 10 mg L^{-1} [36]. Another study also reported the EC-50 values of 17-α ethynylestradiol and estradiol at 3.21 and >10 mg L^{-1}, respectively, for *Navicula incerta* [37]. The authors concluded that estradiol was the least toxic endocrine-disrupting compound. A similar EC-50 value for EST for the microalgal consortium was obtained in the present study as well. However, since the toxicity of estrogen hormones on the growth of microalgae has not been widely reported, it is difficult to make any comparisons.

Diclofenac is found in surface waters at very low concentrations at ng L^{-1} to pg L^{-1}, which are not high enough to cause acute toxic effects. Even though the data for toxicity vary for different pharmaceutical drugs, DCF was found to have the highest acute toxicity within the class of NSAIDs [38]. In addition, it has been reported in some studies that DCF exhibits phytotoxicity and inhibition of algal reproduction to different species of microalgae, but only at very high concentrations. Cleuvers 2003 [39] reported the EC-50 values of DCF for algal species *Desmodesmus* at 72 mg L^{-1}, which is much higher than the concentrations in which it occurs in the aquatic environment. Schulze et al. 2010 [40] reported the EC-50 of DCF for green algae *Scenedesmus vacuolatus* as high as 48.1 mg L^{-1}. A study by Schmitt-Jansen et al. 2007 [41] for algal toxicity reported that, with time, the toxicity of DCF was elevated to 70% inhibition of reproduction, even though the residual concentrations were reduced to 4% of the initial concentration. This can be explained by the higher toxicity of the transformation products of DCF. However, the present study obtained the value of EC-50 for DCF at 8 mg L^{-1} (Figure 2), which is still higher than environmental concentrations, but lower than those obtained in other studies.

The toxicity of triclosan has been studied concerning aquatic organisms and microalgae in aquatic environments. It has been reported that bacterium, crustacean, and fish had similar sensitivities towards the toxicity of TCS, with EC-50 in the range of 260–390 μg mL^{-1}, while microalgae are more sensitive [42,43]. In a study on microalgae species *Chlorella pyrenoidosa*, it was reported that 45.8–85.0% growth inhibition was observed at different concentrations of TCS, and concentrations above 200 ng mL^{-1} significantly affected microalgal growth [44]. One study reported the absence of toxic effects of TCS on *Chlorella* sp., even at a concentrations of 50 mg L^{-1} [45]. The microalgal consortium used in the present study had an EC-50 value for TCS at 8 mg L^{-1}, which is significantly higher than those reported in the literature. However, it was later seen in the experiment that the consortium could not survive beyond 5 days at 8 mg L^{-1} TCS concentration (Figure 2).

3.2. Drug Adsorption on the Microalgal Surface

3.2.1. Algal Density and Chlorophyll Analysis

The comparison of the optical density (OD) during the adsorption study of the drug-spiked and control microalgae shows that the reading at 680 nm for both had a similar trend. From Figure 3a, it can be seen that no immediate microalgal cell death was observed in the initial 12 h due to the high concentration of the spiked drugs in the growth media. The OD values of the control microalgae increased from 0.205 ± 0.0075 to 0.249 ± 0.003 in 12 h, and the OD value was 0.242 ± 0.002, 0.249 ± 0.0015, and 0.239 ± 0.0024 for EST, DCF, and TCS, respectively.

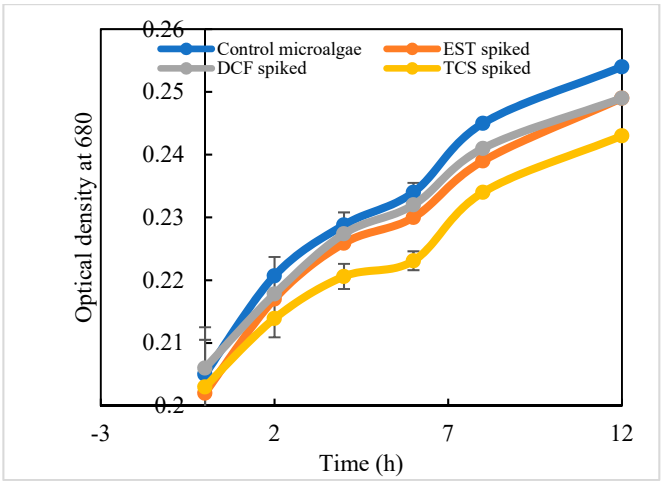
However, in the degradation study, it was observed that, although microalgae were growing in the 16 mg L^{-1} EST- and 8 mg L^{-1} DCF-spiked media, in 8 mg L^{-1} TCS-spiked media after the initial growth until 5 days, the microalgal consortium slowly started showing cell death (Figure 3b). The cells were completely dead by the 8th day. The estimation of chlorophyll content in the EST- and DCF-spiked and control microalgae showed a similar trend of increase in chlorophyll content with time during the experimental period, unlike TCS spiking, where the chlorophyll content declined from the 5th day onwards (Figure 3c).

From the results of the independent *t*-test and ANOVA, it was seen that for EST- (p value = 0.60) and DCF- (p value = 0.78) spiked media, the growth of the microalgae was not significantly different from the control microalgae, whereas for the TCS (p value = 0.0003) spiking, there was significant variation due to the spiking.

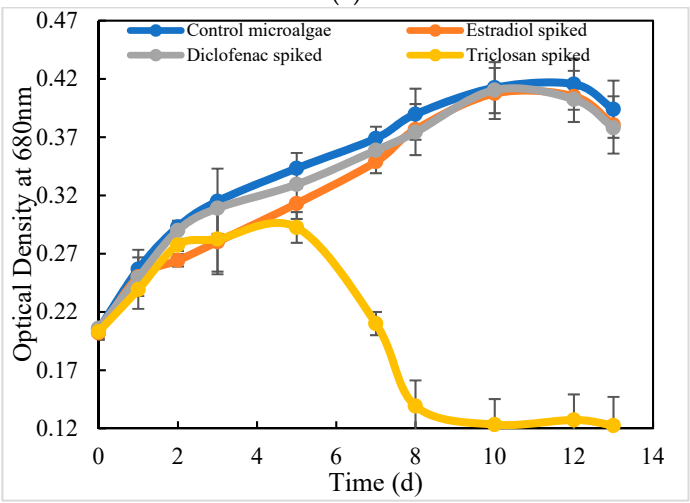
3.2.2. Analysis of the Drug Adsorbed on Microalgal Cells

Adsorption is expected to be a rapid process taking place within a short time. For the study of adsorption as a mechanism for removal of the spiked drugs, microalgal extracts were analyzed by HPLC to determine the content of the drug for the initial 12 h. The extracts did not show any peaks at the expected retention times of the selected drugs. Even if any spiked drugs were in the extracts, they were below the detection limits of HPLC, implying a very small amount. Thereby, it can be implied that adsorption was not the primary mode of removal of the three selected drugs from the spiked media in the current study.

It has been reported in literature that the chemical structure of the ECs affects the adsorption onto the microalgal cells. ECs that are hydrophilic in nature repel the microalgal cell surface, whereas the hydrophobic and cationic ECs are actively attracted to the cells' surface [46]. Thus, in the literature, a high degree of variation is reported in the efficiency of this process, as the adsorption of ECs is highly specific to the structure and properties of the drugs (hydrophobicity, functional groups, surface chemistry, etc.) [47]. Environmental conditions and seasons also influence the adsorption of compounds on microalgae [48]. It has also been reported that ECs are adsorbed poorly on algal biomass as compared to activated sludge. In the present study as well, adsorption of the drugs was not observed on the microalgal cells, thus eliminating adsorption as a primary removal mechanism for the three selected drugs.



(**a**)



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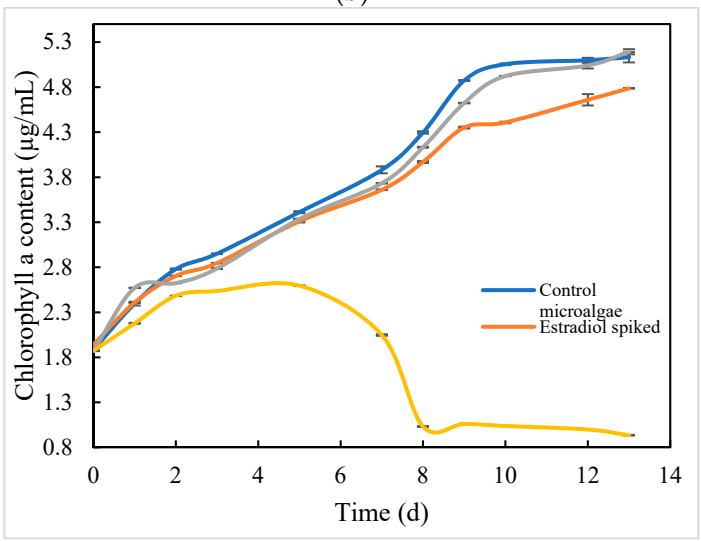


(**c**)

Figure 3. Microalgal growth in presence of spiked drugs: (**a**) optical density at 680 nm for adsorption experiment, (**b**) optical density at 680 nm for degradation experiment, and (**c**) chlorophyll-a content of spiked vs. control microalgae for degradation experiment. Mean of triplicates plotted with ± standard deviation.

Therefore, based on the results of adsorption in the experiment, the study of degradation of the spiked drugs was carried out to assess degradation for removal by the microalgae.

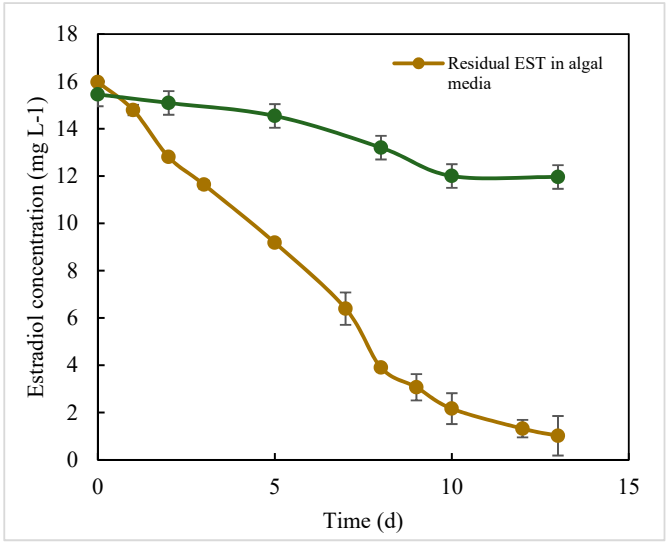
3.3. Analysis of Residual Drug in Culture Media after Degradation

In the present study, the specific removal of EST, DCF, and TCS was 15.55 ± 0.834, 6.13 ± 0.136, and 6.28 ± 0.426 mg g^{-1}, respectively. The drug concentration in the media and the microalgal growth are negatively correlated to each other for the spiked drugs. With the increase in microalgal growth, a decrease in the residual concentration of the EST and DCF in the media was observed. However, it was not the case with TCS-spiked media, as it was seen that even though the specific removal of TCS was more than DCF, the microalgal consortium was not able to survive in the presence of a high concentration of the TCS beyond 5 days. For the three tested drugs, it was seen that the abiotic reduction in the drug concentration was slow as compared to the rapid action of the microalgal consortium. Significant amounts of the drug were removed from the media by the microalgae only.

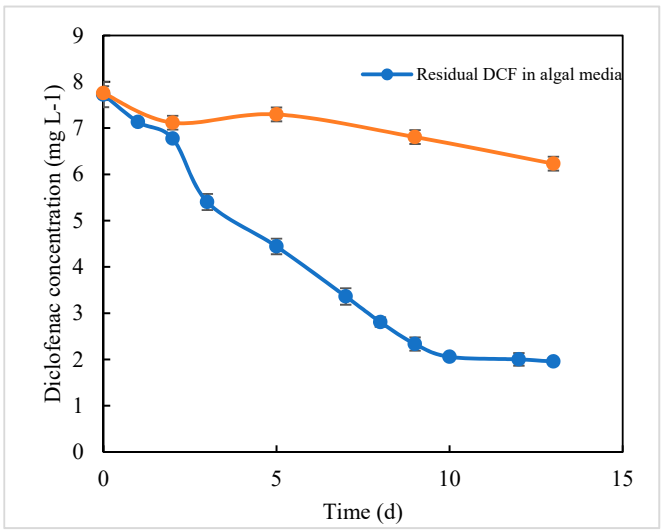
Algal removal mechanisms for ECs have been investigated, and the microalgal biotransformation of many PPCPs has been reported [49]. Previous studies have demonstrated that the microalgae exhibited a compound-, species-, and environment-dependent removal efficiencies of different ECs in wastewater. Some compounds like natural hormones are reported to be easily degraded by microalgae, while synthetic hormones appear to be more resistant to removal [21]. However, it is essential to also point out that many researchers have found that the transformation and degradation processes of toxic organic pollutants can vary widely between different microalgal species, depending on cell size, cell wall composition, and enzyme systems of the species, amongst other factors [50].

In the present study, the EST concentration was monitored daily in the drug-spiked media with microalgae, and it was also compared with the concentration of the drug in the negative control, without any microalgal consortium. From the results of the present study, it can be seen that the degradation of EST by microalgae was high in 8 days within the exponential growth phase of the microalgae, and up to 75.5% of the initial 15.97 ± 0.0219 mg L^{-1} EST was removed from the culture media (Figure 4a). At the end of the experiment, when the stationary phase of growth was observed, up to 91.73% of EST was removed from the media with the final EST concentration in the media at 1.018 ± 0.0175 mg L^{-1}. On the other hand, in the abiotic control sample with no microalgae, the final EST concentration at the end of 13 days was 11.96 mg L^{-1} (±0.0245). In the presence of the microalgal consortium, the removal rate of EST was much higher as compared to the abiotic degradation in the control sample with no microalgae.

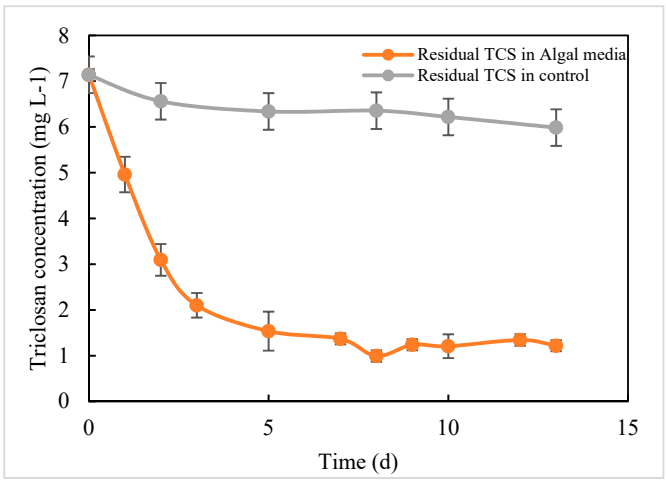
Microalgae in monoculture have been observed to have the ability to remove estrogenic steroid hormones in wastewater via uptake and biotransformation, but the removal efficiencies are species dependent [51]. Sorption, direct-photolysis, and algae-mediated biotransformation have been evaluated as possible removal mechanisms, with maximum removal achieved by biotransformation [52]. A study done to assess the removal of 17β estradiol in algae-based photo-bioreactor showed >93.75% removal of the hormone under favorable seasonal conditions. Under harsh environmental conditions as well, the consortium was reported to have removed 50% of the hormone. *Chlorella* and *Scenedesmus* were reported to be stable and active species during the entire operation [53]. The authors also concluded that high community diversity and evenness have beneficial implications for micropollutant removal.



(**a**)



(**b**)



(**c**)

Figure 4. Drug degradation by microalgae: (**a**) estradiol, (**b**) diclofenac, and (**c**) triclosan.

There are not many studies reporting the efficacy of the microalgal consortium for the removal and mechanism of estrogen hormones. However, some results are there for the removal of these compounds by different species of microalgae in a monoculture. A study by Ruksrithong et al. 2019 [54] investigated the removal of estrone (E1) and 17β estradiol (E2) in synthetic wastewater by *Chlorella vulgaris* and *Scenedesmus obliquuts* in a batch experiment. It was reported that *S. obliquus* removed E1 and E2 by 91% and 99%, respectively, whereas *C. vulgaris* removed E1 and E2 by 52% and 99%, respectively. The biodegradations of E1 and E2 by *S. obliquus* were reported to be 77% and 97%, respectively, *C. vulgaris* degraded E1 and E2 by 38% and 98%, respectively, and the remaining removal was due to adsorption of the drug on the cell surface. Therefore, it was concluded that the predominant mechanism for E1 and E2 removals was biodegradation, while adsorption was considered as a minor one. These results are similar to those obtained in the present study regarding adsorption of EST on the microalgal surface. Rapid removal and biodegradation of 17β estradiol and diethylstilbestrol by *Raphidocelis subcapitata* was observed in a study [55]. The microalgal species was exposed to single and mixture treatments for 96 h and showed 74.6% removal, out of which 72.9% was reported to be from biodegradation. The rapid and strong ability of the microalgae to remove EST by biodegradation was also obtained in our study, as we did not observe simple adsorption on the cells. Therefore, adsorption as a major removal mechanism for estrogens can be ruled out.

Wang et al. 2017 [36] investigated the removal and biotransformation of 17β estradiol and 17α-ethinylestradiol by six microalgal species—*Chlamydomonas* sp., two isolates of *Chlorella* sp., *Scenedesmus quadricauda*, *Selenastrum capricornutum*, and *C. vulgaris*. It was observed that the biological removal of estrogen was an incubation time- and species-dependent process. All the microalgal species investigated were capable of removing the estrogens, with *S. capricornutum* being the most effective. Additionally, it was observed that the adsorption percentage was very low as compared to the total biological removal percentages in all microalgal species. Most of the studies found that adsorption was a minor removal mechanism for estrogen hormones. However, it has also been reported that some algal species show up to 70–80% adsorption after 7 days [56]. While testing *S. capricornutum* and *Chlamydomonas reinhardtii* for biodegradation of β estradiol and 17 α-ethinylestradiol, the authors attributed 42% and 54% of removal to biodegradation processes, respectively, while the rest was due to adsorption.

In the present study, DCF removal was 74.68% with the final concentration in the media decreased to 1.95 ± 0.0092 mg L^{-1} from 7.72 ± 0.271 mg L^{-1} (Figure 4b). The highest removal was observed during the exponential growth phase of the microalgae. While in the abiotic control, the DCF concentration was reduced from 7.75 ± 0.0079 mg L^{-1} to 6.23 ± 0.009 mg L^{-1} in 13 days of the experimental duration. This shows that the reduction in the concentration of DCF was due to the biological action of the microalgae, and abiotic degradation was not significant. There is very limited information available about the removal of DCF by microalgae. However, in a previous study, removal of DCF at 25 mg L^{-1} using different microalgal strains *Chlorella sorokiniana*, *C. vulgaris*, and *Scenedesmus obliquus* in bubble-column photo-bioreactors with operating volumes of 250 mL was reported [33]. Unlike our results, the authors reported that in the presence of DCF, there was a higher biomass concentration and higher growth rate compared to the control of each strain, although the addition of DCF resulted in a larger lag phase for the spiked microalgae. DCF removal efficiencies of 29.99% by *C. sorokiniana*, 21.58% by *C. vulgaris*, and the highest of 79.09% by *S. obliquus* were determined during the batch culture. The authors reported the quickest removal rate for *C. sorokiniana* due to the highest growth rate ($r = 0.9988$ d^{-1}). Growth rates in their study with DCF spiking were higher than those reported by other authors for the tested microalgal species at similar growth conditions without DCF (0.72 d^{-1} for *C. sorokiniana* and 0.38 d^{-1} *C. vulgaris*). This was explained due to the use of DCF as an organic carbon source by the microalgae. Enhanced growth was not observed in the present study.

In the present study, up to 78.4% TCS removal was observed within 5 days, as the concentration decreased from 7.13 ± 0.006 mg L^{-1} to 1.53 ± 0.0127 mg L^{-1} (Figure 4c). In the abiotic control, it was seen that TCS concentration was reduced from 7.14 ± 0.0055 mg L^{-1} to 5.985 ± 0.015 mg L^{-1} at the end of 13 days of the experiment. However, the high concentration of 8 mg L^{-1} of TCS was seen to have a toxic effect on the microalgae and it was not able to sustain growth further (Figure 3). Cell death was observed within the exponential growth phase and no further removal of TCS was observed. As reported in some studies, microalgae have been reported to be highly sensitive to TCS and such high concentrations for TCS have not been tested. Earlier studies have been done for TCS removal by microalgae have tested environmental concentration spiking in the range of ng-µg mL^{-1}. A high removal rate of up to 99.7% was observed during the treatment of 400 µg L^{-1} TCS by the three common freshwater microalgae including *C. pyrenoidosa*, *Desmodesmus* sp., and *S. obliquus*. The removal was attributed to cellular uptake and biotransformation [57]. The same group also investigated the TCS removal at a range of concentrations from 100 to 800 ng mL^{-1} by *C. pyrenoidosa*. Removal percentage of 77.2% by biodegradation was obtained for 800 ng mL^{-1} in 96 h [44]. *Nannochloris* sp was reported to have 100% removal of TCS in 7 days [58]. *Geitlerinema* sp. and *Chlorella* sp. were shown to degrade 82.10% and 92.83% of 3.99 mg L^{-1} of TCS, respectively, in 10 days [45]. Microalgae grown with different supplements like phenol, propane, etc. in growth media have been reported to degrade 95–100% of 5–10 mg L^{-1} TCS [59,60]. It has also been reported that some algal strains showed less susceptibility to TCS when grown in complex and mineral salt media [61]. Since in the present study no additional enrichment of media was done, the microalgae did not sustain growth beyond 5 days. However, degradation of the spiked TCS was still observed in our study.

A study done to remove 25 ECs, including TCS and DCF, using mixed microalgal strains in an algal-based wastewater treatment system reported that DCF removal efficiency was above 82% at HRT of 4 days and above 92% at HRT of 8 days during the warm season [48]. In another study, the removal of many ECs (including EST, DCF, and TCS) by *C. sorokiniana* grown on urine, anaerobically treated black water, and synthetic urine was investigated in batch experiments [31]. The mixture of contaminants was at environmental concentrations, and 40–60% DCF removal was observed, including the abiotic batches. Therefore, it was proposed that photo-transformation and photolysis led to the removal of DCF. Apart from biotransformation, photolysis can be a major removal mechanism in microalgal treatment systems as these processes use illumination for algal biomass growth, which in turn also increases the removal of many of the light-sensitive ECs.

Biodegradation is a fundamental way by which algae can eliminate estrogenic hormones quickly. Both 17 a-estradiol and 17 b-estradiol can be bio-transformed into estrone by microalgae, which can then be transformed into estriol via hydroxylation and degraded into other unknown compounds [52]. The microalgae *Selenastrum capricornutum* and *Chlamydomonas reinhardtii* were shown to biodegrade the hormones 17 b-estradiol and 17-ethinylestradiol [56]. Peng et al. 2014 [21] identified reduction (hydrogenation), hydroxylation, oxidation (dehydrogenation), and side-chain breakdown as the primary mechanisms involved in the microalgal transformation of progesterone and norgestrel.

Nannochloris sp. eliminated triclosan completely in 7 days, and this was attributed to microalgae-mediated absorption and photolysis [58]. However, biotransformation was primarily responsible for TCS removal by *M. aeruginosa*, with methylation being a prominent biotransformation pathway, and with 51% of the removed TCS being changed into methyl-triclosan [62]. DCF removal differed significantly amongst microalgae species [33]. *Scenedesmus obliquus* eliminated 79.09% of the DCF in 9 days, whereas *C. vulgaris* only removed 21.58%, owing to biodegradation.

The biodegradation of ECs by microalgae has been documented in the literature over the last five years as the major removal mechanism. In microalgal-based systems, microalgae can also actively participate in the biodegradation of organic contaminants. Microalgal enzymes can metabolize a wide spectrum of xenobiotic compounds by three stages [36].

Phase I involves the conversion of lipophilic xenobiotics into more hydrophilic molecules, which are easier to excrete. Cytochrome P450, which are microsomal heme-thiolate proteins anchored in membranes, normally catalyze the first stage of detoxification [63]. Phase II is distinguished by the inclusion of hydrophilic molecules to assist excretion. Glutathione (GSH] or glucuronic acid can be conjugated with xenobiotics and metabolites from phase I, which is catalyzed by glutathione S-transferases (GSTs) or glucosyltransferases [64]. In phase III, xenobiotics are compartmentalized in vacuoles or cell wall fractions [65]. Microalgae are thus referred to as "green livers" for environmental detoxification of pollutants since their ability to detoxicate xenobiotics is similar to that of a mammalian liver [66].

One of the difficulties in screening microalgae for possible EC biodegradation is that the enzymes involved may not be active at the time of screening, and the microalgae may require a certain concentration of the EC before the degrading enzymes are activated. The cell pays a metabolic price for the creation and maintenance of these enzymes, which comes at the expense of growth and reproduction. Following exposure to four different antibiotics, Aderemi et al. 2018 [24] discovered that the microalga *Raphidocelis subcapitata*'s cellular energy budget and growth rates were dramatically lowered. The authors concluded that the reduced cellular energy budget was due to the cells' synthesis of superoxide dismutase in response to antibiotic treatment. Therefore, prior to conducting any biodegradation potential screening, pre-acclimation of the microalgal strains to sub-toxic doses of the contaminant of concern may be necessary. Microalgae adapted to contaminants have been found to have improved photosynthesis, growth rates, metabolic activities, and/or other cellular activities, e.g. [23,67]. When *C. pyrenoidosa* was pre-exposed to the antibiotic cefradine, its removal effectiveness was enhanced, according to Chen et al. 2015 [68]. Similarly, Xiong et al. 2017 [46] discovered that when *C. vulgaris* was pre-acclimated to the antibiotic, levofloxacin biodegradation rose considerably. Increased xanthophyll pigment synthesis was mentioned by both authors as part of the acclimatization mechanism. These pigments operate as antioxidants, membrane stabilizers, and radiation protectors [46].

Another issue with biodegradation is that, though the breakdown products are usually less harmful to aquatic biota and human health, this is not always the case. As a result, isolating, identifying, and screening the breakdown products for toxicity and fate is a critical step in creating microalgal biodegradation treatment systems.

Microalgae may also potentially aid in the biodegradation of ECs by bacteria when present together as a consortium. Microalgae can also help bacteria degrade ECs by releasing dissolved organic matter (DOM), which can offer the essential substrates for bacterial co-metabolism of the pollutant. However, the specific mechanism for this improved biodegradation is unknown. Microalgae either released exudates, such as enzymes or oxygen, that helped disintegration, or microalgae took up the compounds, resulting in increased biodegradation. Relationships between microalgae and bacteria in terms of EC degradation, on the other hand, may not always be helpful, as both are capable of suppressing each other, depending on the species.

The SEM analysis shows that the microalgal cells in the EST-spiked (Figure 5b) media show wrinkling and deformation in the cell wall, not seen in the control microalgal cells (Figure 5a). The spiked media grown cells show deformation in the cell shape. DCF-spiked cells (Figure 5c) appear to be more clustered than the control cells, whereas the TCS-spiked cells (Figure 5d) show complete cell disruption and a lot of cell debris. The microalgal cells of the control without any drug spiking are smoother. There is lack of reports regarding morphological changes in the microalgal cells due to pharmaceutical drugs. However, one study reported the changes in the ultrastructure of microalgal cells observed under TEM when exposed to TCS. It was observed that for the algal cells treated with TCS, the chloroplast membrane got damaged, the inner stroma spilled out, and the ordered triple-layered lamellae of the chloroplast became uneven after 96 h of exposure. Cell lyses and organic material release into the aquatic environment were also observed. This indicated that cell chloroplasts were the main targets of TCS in microalgae, thereby affecting

photosynthesis, reducing the energy production, and leading to growth rate inhibition along with an adverse effect on the of removal and biodegradation of TCS [44].

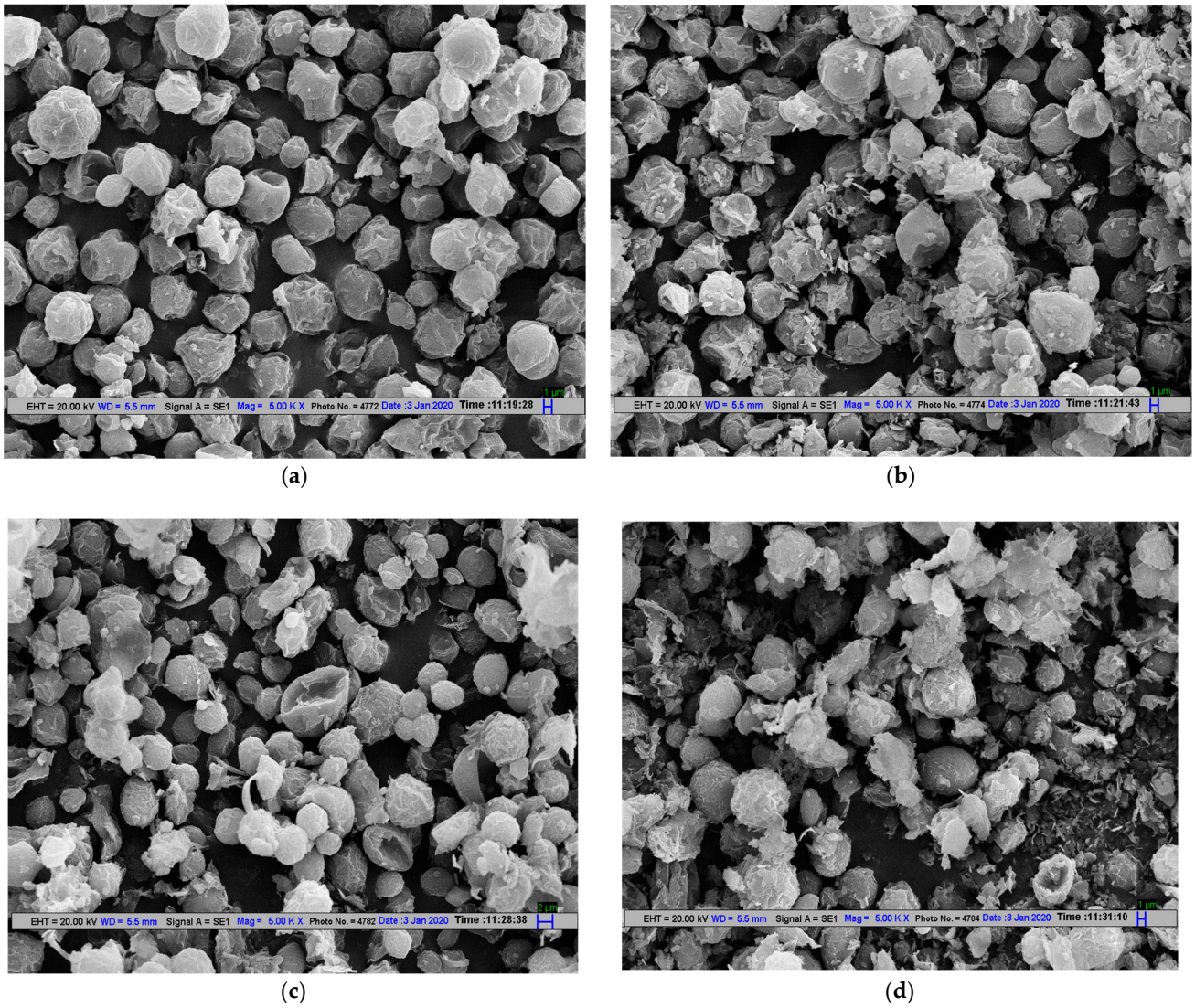


Figure 5. SEM images showing morphology of microalgal cells in (**a**) control vs. (**b**) drug spiking with estradiol, (**c**) diclofenac, and (**d**) triclosan. All images were taken at 5.00 k resolution.

4. Conclusions

Results obtained from these experiments indicate that the microalgal consortium can tolerate the presence of environmentally high concentrations of pharmaceutical drugs as their growth was observed in these experiments. Additionally, the consortium was further able to remove the spiked drugs from the culture media. The main removal mechanism observed in the present study was biodegradation, whereas adsorption on the microalgal cell surface was not significant. Microalgal removal of ECs can be achieved in a short time as compared to their natural, abiotic degradation in the environment. The positive results obtained in the study open up promising prospects for the application of the microalgae-based treatment for the removal of a variety of pharmaceuticals in addition to removing the excess nutrient load from wastewater. Combining the algal process with some chemical process can further accelerate the targeted removal of many ECs. In-depth studies are required to better understand and elucidate the chemical reactions, the degradation products, and to decipher the exact removal mechanism by microalgae.

In addition, the degradation of products of ECs needs in-depth investigation for their potential toxic effects in the environment.

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