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A hybrid process for 2,4-dichlorophenoxy acetic acid herbicidal treatment and its microbial identification by MALDI-TOF mass spectrometry

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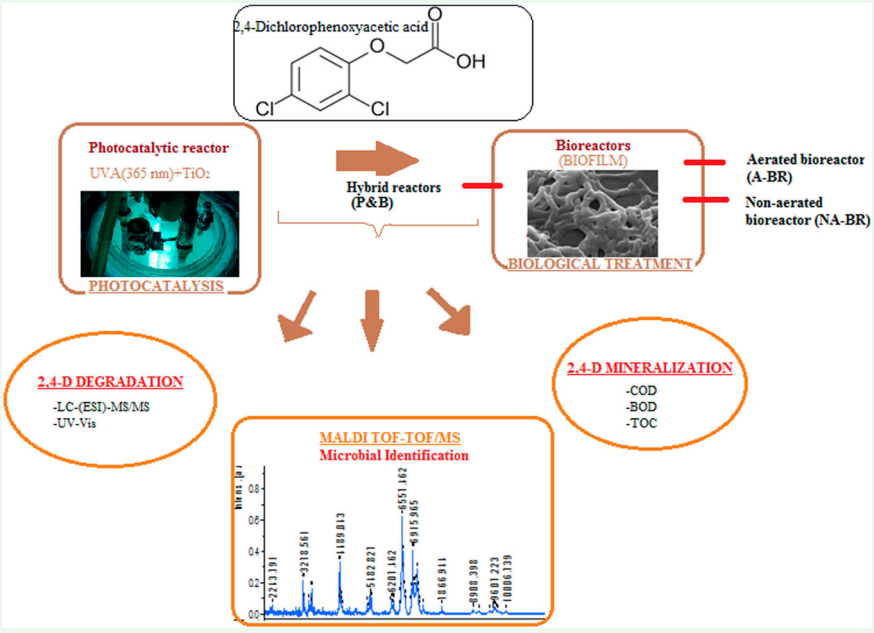
ABSTRACT

The feasibility of coupling photocatalysis and a biological treatment to remove a herbicide – 2,4-dichlorophenoxy acetic acid (2,4-D) – from pure water was examined using batch experiments following three protocols: aerated (A-BR) and non-aerated biodegradation (NA-BR) alone, and intimately combined photodegradation and biodegradation (P-B). In view of a subsequent biological treatment, 15 and 180 min irradiation times were chosen in accordance with spectrophotometric and LC-MS/MS results that indicated the decrease in the COD/TOC ratio during photocatalysis. Pre-treatment led to a quick decrease in concentration of 2,4-D and COD during the biological process: a 78.79 ± 0.30% COD removal and 38.23 ± 3.12% 2,4-D elimination was measured after 5760 min in A-BR, and 80.89 ± 0.81% COD and 81.36 ± 1.37% 2,4-D removal was achieved after 2880 min in P-B. For species identification using matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-TOF/MS equipment, *Aeromonas eucrenophila*, *Stenotrophomonas acidaminiphila*, *Ralstonia pickettii*, *Sphingobacterium multivorum* and *Acinetobacter towneri* were identified with high accuracy, and they play important roles in the degradation of 2,4-D.



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2,4-dichlorophenoxy acetic acid; biodegradability; biological treatment; photocatalysis; MALDI-TOF-MS

1. Introduction

The last two decades have witnessed a sensational surge in concern about water and wastewater treatment as a way to protect water sources, the health of living beings and the environment, against the consequences of pesticide contamination [1]. In many regions of the world, and especially in Turkey, 2,4-dichlorophenoxy acetic acid (2,4-D) has been one of the most widely used chlorinated phenoxy-alkanoic herbicides, especially in potato, grain and corn production. It is used for

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controlling broad-leaf weeds in a variety of agricultural activities and gardening due to its high efficiency and low cost [2]. 2,4-D is considered moderately toxic (class II) by the World Health Organization (WHO), and its carcinogenicity, neurotoxicity and endocrine disruptive potential cannot be neglected [2,3]. Since this compound is mostly degraded to the anionic form, which exhibits high water solubility (667 mg L^{-1}), high mobility and is long lasting, its continuous use may cause soil percolation, and surface and groundwater contaminations. Moreover, 2,4-D is often detected in surface (≈6 µg L^{-1}) and drinking water (2.2–3.2 µg L^{-1}) sources [4]. The half-life of 2,4-D in the environment is relatively short, averaging 10 days (1–6 weeks) due to its susceptibility to biological treatment in soils [5] and less than 10 days in water. The regulatory standard for 2,4-D in drinking water is 0.1 and 70 µg L^{-1} as per the Turkish standard [6] and the U.S. standard [7], respectively.

The main problem is that it is not feasible to treat water polluted with pesticides with classical biological treatment methods, especially those based on pure strains of bacteria. The toxicity of the microorganisms involved in these processes, the long treatment time and the presence of halogens in the molecular structure of 2,4-D that renders it highly persistent and resistant to biodegradation [8,9]. The most recent studies – including this study – conducted on biodegradation used microbial mixtures without using a pure culture to imitate realistic conditions and maintain microbial activities over a wider range of conditions that could affect the rate of biodegradation [8,10]. Moreover, because of the biorefractory nature of chlorophenoxy herbicides, a great deal of attention has been given to the extension, development and advancement of innovative approaches, ideas or designs. These innovative developments focus on enhancing the efficiency of well-known technologies – or combining them with sustainable, cheaper environmentally friendly treatment methods – to reduce costs or eliminate other disadvantages [11]. In recent years, by producing *OH radicals through chemical, photochemical, photocatalytic and electrochemical reactions, advanced oxidation processes (AOPs) have become a promising technology to eliminate pesticides from water systems [1]. Unfortunately, the AOPs – like photocatalysis – are limited due to the high cost and energy required to run the process and to achieve complete mineralization. In addition, these processes can produce more toxic and/or bio-recalcitrant intermediate products, and there is a lack of knowledge on the process design and operation of large-scale reactors based on this principle [9,12]. Recently, in order to degrade recalcitrant compounds, reduce the toxicity, treatment time and economic/energy costs, employing AOPs as a pre-treatment method in combination with biological treatment has been recommended [13,14]. The most common arrangement is pre-treatment with AOPs to degrade pollutants to make them more easily converted and oxidized through biodegradable methods. Biological processes are then used as a second step to complete mineralization and oxidation of intermediate products [15]. This combination model is beneficial in order to protect microorganisms from UV irradiation and hydroxyl free radicals during photocatalysis.

In published scientific literature, there are a limited number of reports the potential of coupled photocatalytic-biological treatment [8,12,15–17]. However, this is the first comprehensive study on the hybrid treatment of 2,4-D herbicide using a mixed-culture biofilm system and the detailed identification of bacterial isolates for all the biological systems. Matrix-assisted laser desorption/ionization, time of flight, mass spectrometry (MALDI-TOF/MS) is a method for determining the genus, species and even sub-species of bacterial isolates for the technique, chosen because of its basic sample preparation steps, low financial cost per analysis and rapid acquisition time [18]. In this work, firstly, the optimal time ratio was determined for the hybrid process by assessing degradation, oxidation and mineralization results of the 2,4-D; then the biodegradability of the residual 2,4-D solution was evaluated. Secondly, residual 2,4-D concentrations and chemical oxygen demand (COD) were analysed in three batch mode-operated biological reactors: aerated (A-BR), non-aerated (NA-BR) and hybrid reactors (P-B). Finally, MALDI-TOF-TOF/MS techniques were used to evaluate the impacts of 3 different bioreactors for 2,4-D degradation on the community structure before and after the biodegradation.

2. Materials and methods

2.1. Chemicals

All chemicals used in the investigation were used without further purification. Titanium (IV) oxide nano powder (AEROXIDE® P25 ≥99.5%, 21 nm, 35–65 m^2 g^{-1} (BET)) was used as a photocatalyst (Sigma Aldrich) due to its high performance and availability in photocatalytic degradation reactions. Commercial grade Amin EXT 500 SL (equivalent to 500 g L^{-1} of 2,4-D) 2,4-D amine salt ($C_{10}H_{13}Cl_2NO_3$, MW:266.12 g mol^{-1}) was supplied by the Agrofarm® Company. Plate Count Agar (PCA) was obtained from Merck (Germany) as a solid medium for microbial cultivation. Other chemicals: NaOH, H_2SO_4 (essay 97%), NH_4Cl, $CaCl_2{\cdot}2H_2O$, $FeCl_3$, $MgSO_4{\cdot}7H_2O$,

$MnCl_2{\cdot}4H_2O$ and Na_2MoO_4 were obtained from Merck (Germany). All chemicals employed were used without further treatment. All solutions and reaction mixtures were prepared with purified water (Merck Millipore, spec. resistivity: 18.2 MΩ cm).

2.2. Photocatalytic set-up

Photocatalytic experiments were performed in a 4.6 L (operating volume: 1 L) cylindrical, (14 cm D × 30 cm L) batch photoreactor maintained at 22 ± 1°C. The photoreactor was constructed from three parts: (i) an exterior Pyrex glass; (ii) a Pyrex glass thimble, with the head part fitted to the outside container to form a gastight seal so that running water can be passed through the thimble to cool the reaction solution and (iii) an empty quartz chamber in which a Philips PL-L UVA 36 W lamp (315–380 nm; 110 μW cm^2) was placed. The reactor was also equipped with a control system, a water level sensor system, and water inlet–outlet and gas inlet opening to supply air from a diffuser system with a capacity of 3.5 L min^{-1} during the experiments. The reactor was wrapped with aluminium foil to prevent UV ray penetration. For irradiation experiments, the desired 2,4-D concentration was diluted from a 100 mg L^{-1} stock solution in amber-glass vessels and they were prepared daily. The system was stirred and aerated in order to increase the oxygen transfer to the solution following the addition of the TiO_2 for at least 30 min in the dark and allow the system to reach equilibrium in case of adsorption. This time was determined so that, under stirring in the dark, no more herbicide molecules could be adsorbed by the photocatalyst. The UV light was turned on to irradiate the solution and the first sample was taken ($t = 0$). The photo-oxidized solution was filtered using Merck Millipore filters (0.45 μm). The photocatalytic experiments were repeated three times to check the reproducibility of the experimental results. The mineralization experiments were performed in duplicate for 1440 min.

2.3. Biological set-up

The set of biodegradation experiments were conducted in a FerMac 200 (working volume: 500 mL; 205 × 390 mm) batch bioreactor, and a 3 L beaker was used as the second parallel batch bioreactor. The bioreactors contained 100 mL of 2,4-D herbicide and 400 mL of bacterial medium (BM-2,4-D medium, equal in total to 50 mg L^{-1} of 2,4-D herbicide). The biodegradation experiments were performed in duplicate. An aerated bioreactor (A-BR) and hybrid reactors (P-B) were stirred at 150 rpm and air bubbled through them at a rate of 3.5 L min^{-1} during the experiments by using two separate magnetic stirrers (Rocker HP 220, Heidolph MR-Hei standard D-91126), an air pump (Hailea Aco 6603) and a diffusor to saturate the water with oxygen. In addition, a digital thermometer was used to continuously monitor the temperature in the reactors to keep it constant at 24 ± 1°C. Three protocols were employed i.e. photocatalysis alone, aerated and non-aerated biodegradation (A-BR, NA-BR), and hybrid reactors (P-B), to evaluate 2,4-D degradation in batch experiments.

2.4. Bacterial medium

The bacterial medium consisted of 3.6 mM of NH_4Cl, 45.0 μM of $CaCl_2{\cdot}2H_2O$, 0.6 μM of $FeCl_3$, 4.2 μM of $MgSO_4{\cdot}7H_2O$, 1.6 μM of $MnCl_2{\cdot}4H_2O$ and 24.3 nM of Na_2MoO_4 (Merck, Germany) in MilliQ (Millipore, Merck) water [17]. After the pH of the medium was adjusted to a range from 7.0 to 7.2 with 1 M of phosphate buffer, the nutrient solution was autoclaved and cooled for 15 min at 121°C.

2.5. Inoculum

Activated sludge was obtained from a secondary clarifier sewage treatment plant in Bolu, Turkey, and frozen in 15 mL aliquots at −20°C in a 1:1 mixture of activated sludge and 50% glycerol (v/v). Prior to the reactor inoculation, the aliquot was thawed, centrifuged at 5000 rpm for 5 min, and 15 mL of the supernatant was decanted and replaced with fresh bacterial medium. This washing process was repeated three times to remove the glycerol from the inoculum.

2.6. Analytical methods

COD was measured by using COD test kits (Test Kits No: 1.14541 from Merck, Germany) with a UV spectrophotometer (Pharo 100). A 3 mL sample was measured after treatment for 120 min at 150°C in the thermoreactor (Merck Spectroquant TR 320). Total organic carbon (TOC) was analysed with a Shimadzu TOC-L using the NPOC method (680°C, 150 mL min^{-1}, with an injection volume of 20 ml, a spray gas flow of 80 ml, and a spray time of 1.30 min). The pH and temperature measurements of all samples from the photocatalytic reactor were measured using a pH meter (Orion Star A329 Thermo Scientific) and a pH probe (8107UWMMD ROSS pH/temperature electrode). A Liquid Chromatography–Mass Spectrometry–Electrospray Ionization instrument was used (Thermo TSQ Quantum Access Max LC–(ESI)–MS/MS) to verify the degradation of the 2,4-D into intermediate products, to identify the metabolites and to determine the residual herbicide concentration using

the AOAC Official Method 2007.01 (Mobile phase A: 5 mM of ammonium formate, 0.1% of formic acid (95:5 Water:MeOH) and Mobile phase B: 5 mM of ammonium formate, 0.1% of formic acid (5:95 Water: MeOH)). SEM (Scanning Electron Microscopy) images were taken to observe the biofilm formation in the bioreactor systems. SEM analyses were performed using FEI Quanta FEG 250 Scanning Electron Microscopy. Microorganism species formed and disappearing at the entry and exit of the bioreactor was determined by placing them in AutoFlex MALDI-TOF-TOF-MS (Bruker) using BioTyper (Version 3.1) software and a reference database (in positive linear mode with a laser frequency of 50 Hz). MS signals were obtained using the manufacturer's automated method – MBT_FC.par – for each sample in the positive linear mode and from the 2 to 20 kDa *m/z* range.

2.7. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) profile acquisition and bacterial identification

Firstly, to prepare samples by the direct transfer (DT) method, the biological material (single colony) was directly applied as a thin film on a MALDI spot plate taken from an aqueous PCA petri dish that was pre-incubated overnight. HCCA (*α*-cyano-4-hydroxycinnamic acid) was used as a matrix, and a composition of 50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid was used as a standard solvent. One µL of HCCA matrix solution was coated onto the biological material. The spotted layers were left to dry at RT. Subsequently, the target layer was loaded onto a MALDI-TOF/TOF-MS instrument and the mass spectrum was automatically formed from the samples and then analysed using an AutoFlex mass spectrometer connected to the MALDI instrument. For isolates with scores less than 2.0 – after the DT method was applied – the *in situ* ethanol/formic acid extraction method was then applied to the layer [19].

MALDI-TOF/TOF-MS profiles taken from bacteria isolates were matched with reference MALDI-TOF/TOF-MS and the Log (score) and related colour code (green, yellow and red) was indicated by the BioTyper. If the Log score was higher than the green colour code, it indicated a high probability description of the species level. If the Log score was between 2.0 and 2.3 (green), it indicated a high probability description at a kind level. If the Log (score) was between 1.7 and 2.0 (yellow), it indicated only a probable kind description, while if the score is less than 1.7 (red), it means that there was no significant similarity between the unknown profile and any of the database. Moreover, consistency categories A, B and C are assigned to descriptions by the Biotyper software that is based on analysing the ten best matches. For category A, in the species consistency, the best match was classified as green by MALDI (species description). For category B, in the kind consistency, the best match was classified as green or yellow. In this category, the conditions for species consistency were not fulfilled. For category C, there was no consistency for either species or kind [20].

3. Results and discussion

3.1. Photocatalytic mineralization performance of 2,4-D herbicide

Before considering the coupling of photocatalysis and biological treatment, optimization studies were conducted spectrophotometrically for governing parameters such as pH (3, 5, 7, 9), TiO_2 concentration (0, 0.5, 1, 1.5, 2 g L^{-1}), initial herbicide concentration (10, 25, 50, 100 mg L^{-1}) and H_2O_2 concentration (50, 100, 150, 200 mg L^{-1}). The maximum removal efficiency was achieved for an initial 25 mg L^{-1} 2,4-D herbicide concentration at a pH of 5, using 1.5 g L^{-1} of TiO_2 and 150 mg L^{-1} of H_2O_2 (data not shown). Spectrophotometric measurements indicated that 15 min of photocatalysis produced the most degraded mixture of products (data not shown). However, the rise that was observed following 15 min gave us every reason to suspect that interference had been occurring. So, in the first step, by using optimal degradation parameters, photocatalytic oxidation experiments were carried out until total 2,4-D elimination by LC-MS/MS – and its total mineralization – in order to get information on the kinetics (Figure 1). The mineralization performance and biodegradability of the photocatalytic treatment of 2,4-D herbicide was constructed as the first stage of a hybrid process in which effluent from the photocatalysis reactor would be fed to the biological reactor for further treatment. The relationship between the photodegradation efficiency of 2,4-D with illumination time was investigated by fixing the pH at 5, using an initial herbicide concentration of 25 mg L^{-1} and a catalyst concentration of 1.5 g L^{-1} of P25 TiO_2. All mineralization studies were completed within 1440 min but they were limited to 200 min to obtain a better image in the graphs. LC-MS/MS analyses confirmed a degradation efficiency of 97.47 ± 0.27% for 2,4-D after 60 min (Figure 1). Djebbar et al. [21] stated that at a pH of 4.2, and 5.10^{-4} M of 2,4-D was rapidly degraded in approximately 100 min. The oxidizing power of the $OH^{\bullet}$ radicals that were generated during photocatalysis was known to be strong enough to completely oxidize 2,4-D to CO_2, H_2O and other mineral acids [22]. As shown in Figure 1, the increase in 2,4-D photodegradation was only 5% from 60 to 180 min. The probable reason could be that, when the irradiation time increased, a large number of small organic molecules were produced and adsorbed

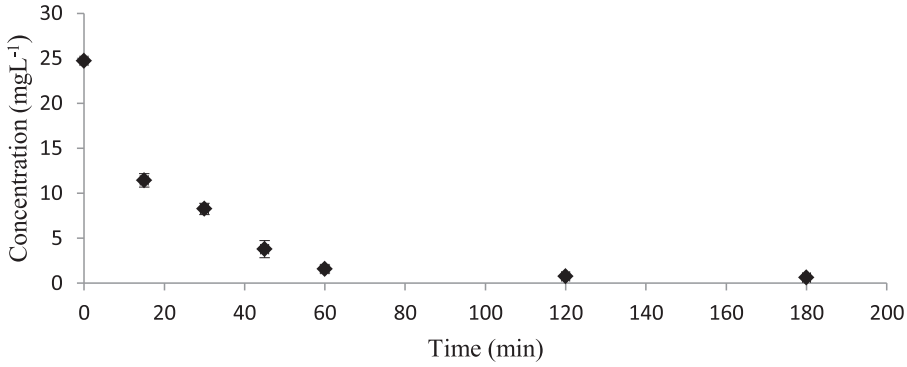


Figure 1. Time-dependent measurements of 2,4-D concentration during the photocatalytic degradation of 2,4-D ($C_o = 25$ mg L^{-1}).

onto the surface of the TiO_2 resulting in decreased formation of $OH^{\bullet}$ radicals that could attack the 2,4-D molecules [22].

Mineralization was investigated through measuring COD and TOC. Decreases in COD during irradiation indicated that 2,4-D was transformed into intermediate products by photo-oxidation, CO_2 and H_2O as the reaction progressed [23]. Approximately 53.04 ± 3.14% of the TOC decrease was obtained at the end of 1440 min photocatalytic experiment. The lowest COD concentration obtained was 20 ± 2.83 mg L^{-1} at 180 min, which also corresponded to a TOC concentration of 13.56 ± 0.77 mg L^{-1} (Figure 2). Based on the repetitive experiments, the maximum COD removal was obtained at 180 min then stabilized that was thought depending on the excess oxygen required by the intermediates formed after this time (Figure 2). After 180 min, COD concentration was 65.52 ± 3.88% of the initial concentration, indicating significant residual organic content. This was also confirmed by the TOC profile. At the end of 1440 min, although 53.04 ± 3.14% of the initial carbon amount in 2,4-D could be transformed into CO_2, photocatalytic mineralization of 2,4-D was completed (Figure 1). This phenomenon can be attributed to the formation of organic intermediates during the period between the degradation of the first molecule and total mineralization. In this study, after degradation of 2,4-Dicholorophenoxy acetic acid ($m/z = 221.0$) with TiO_2/UV, the products formed were 2,4-dichlorophenol (2,4-DCP) (m/z 163.0) in high amounts, 2,4-dichloro-1-methoxy benzene (2,4-DCA) ($m/z = 176.0$) and 4-chlorophenol (4-CP) ($m/z =$ 128.50) in low amounts. Singh and Muneer [24] obtained many intermediates by analysing 2,4-D with GC/MS in the presence of a P25 catalyst: 2,4-dichlorophenol (2,4-DCP), 2,4-dichloro-1-methoxy benzene, benzaldehyde, benzyl alcohol, 3,5-dichlorobenzene-1,2-diol, 4-chlorophenol (4-CP), 4-6-dichlorobenzene-1,3-diol and 3-chlorobenzene-1,2-diol. They indicated that 2,4-dichlorophenol formed as a result of bonding ^{-}OH radicals to the alkyl chain in the molecule and it was the first basic intermediate of 2,4-D. Longer irradiation times were necessary to perform complete mineralization under the given experimental conditions [25].

Biodegradability of photocatalysis products becomes important for the biological stage that will following the current study. Therefore, an increase in oxidation of the target compound (COD decrease) with limited mineralization (low TOC decrease) is a favourable trend to

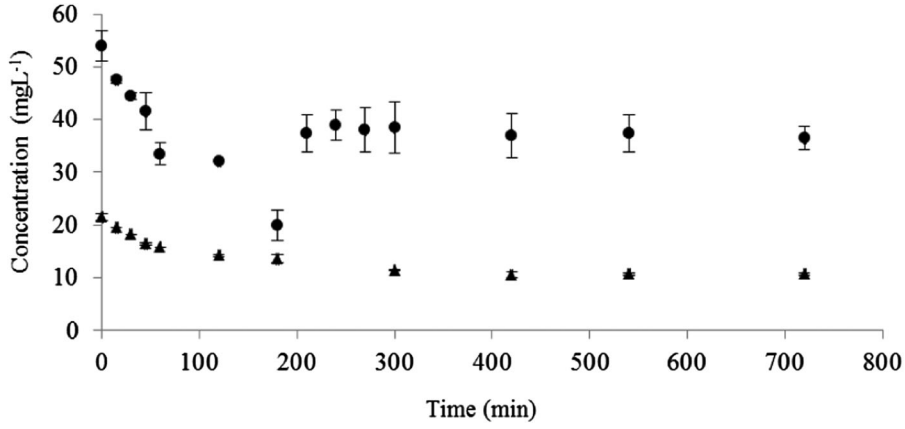


Figure 2. Time-dependent measurement of COD (●) and TOC (▲) during the photocatalytic degradation of 2,4-D ($C_o = 25$ mg L^{-1}).

understand the significant residual organic compounds for microbial cultures [16]. Finally, a favourable trend is a decrease in the ratio of COD/TOC during the photocatalytic degradation of the target compound, in other words, 2,4-D herbicide (Figure 2). COD removal was a maximum where the majority of 2,4-D was eliminated and then afterwards the COD value increased and was stable, probably due to the formation of stable by-products. According to Bouafia-Chergui et al. [26] the reason for incomplete removal of COD is the formation of toxic byproducts due to the photocatalytic treatment. Since the second stage of this study was going to be a biological treatment, oxidizable organic content was important to investigate. As shown in Figure 2, COD and TOC experiments have pointed out that at 180 min the COD/TOC ratio reached a minimum. We concluded that two time points, one determined spectrophotometrically (15 min) during the optimization studies of batch mode photocatalytic experiments and the other determined through measuring COD/TOC (180 min), might produce two different and possibly favourable influents for the biological treatment. From LC-MS/MS measurements we knew that the effluent after 15 min would have 11.43 ± 0.75 mg L^{-1} of 2,4-D, (Figure 1) yet the spectrophotometer measured a minimum for every optimization experiment. The COD/TOC ratio was a minimum at 180 min indicating the maximum degree of reduction for carbon compounds in the system. Since 180 min would clearly provide a more biologically favourable photocatalytic effluent, 15 min produced a more energetically favourable (less irradiation time) photocatalytic effluent.

3.2. Biodegradability performance of 2,4-D herbicide

The biodegradation of process effluent from the 2,4-D solution with an initial concentration of 25 mg L^{-1} at 15 and 180 min – processed by the photocatalytic treatment – is determined by the BOD_5/COD ratio. At the beginning, the biodegradation index (BOD_5/COD) ratio ≈0 while the BOD_5/COD ratio of the process effluent at 15 min was 0.1, and the BOD_5/COD ratio of the process effluent at 180 min was 0.20. Accordingly, because the BOD_5/COD ratio was less than 0.4, it was shown that the photocatalysis solution was poorly suited to biological degradation [27]. Fontmorin et al. [28] stated that the BOD_5/COD ratio was 0.04 before the analysis while it increased to 0.25 after electrocatalysis, indicating a significant increase in the biodegradability of the waste, and while this phenomenon cannot obtain the biodegradability limit, further biological treatment can be promising. In the study, it was considered that the presence of a similar commercial dimethylamine salt could increase general biodegradability. At the end of 180 min, although biodegradation increased in low amounts due to irradiation, it could not reach the limit of degradability. As seen in the COD mineralization graph in Figure 2, it was determined that COD decreased from 65.52% to 35.34%, depending on the formation of stable intermediates after 180 min. It is thought that a microorganism community accustomed to the environment can increase COD mineralization after 180 min. Therefore, although biodegradability of the 2,4-D solution obtained from the photocatalytic process is low, this phenomenon also indicates that the mineralization change of this pollutant should be examined with a mixed microorganism culture accustomed to 2,4-D herbicide at a concentration of 25 mg L^{-1}. The COD value was 20 ± 2.83 mg L^{-1} on average at 180 min, and it was seen that because it had a low amount of residual organic substance, it was possible to completely treat 25 mg L^{-1} of 2,4-D with a photocatalytic process. Thus, because the 15 min effluent value selected for the photocatalytic treatment (average COD amount: 47.5 ± 0.71 mg L^{-1}) included a sufficient amount of waste substance for a combined photocatalytic and biological treatment in that time, the similarity of treating it with a hybrid method was seen [29].

3.3. Biological treatment performance on 2,4-D degradation

To optimize efficiency, it is very important to determine the optimum time for processes where photodegradation and biodegradation are present together. If the photolysis time is too low, biodegradation will be inefficient because the pollutant will not be biodegradable enough. If it is too long, the process will not be economical because of the high cost of the photolysis process [30]. Recent research, including this study, has been carried out on biodegradation using mixed microbial mixtures (without using pure cultures) to mimic real conditions and maintain microbial activity over a wider range of conditions that will affect the rate of biodegradation [8,9].

Although 25 mg L^{-1} 2,4-D was obtained as an optimum 2,4-D concentration at photocatalytic process, 50 mg L^{-1} of 2,4-D concentration was selected to degrade in biological reactor system in order to increase the organic pollutant concentration for microbial degradation. The change in removal efficiency of 2,4-D at the beginning and at day 4 was 9–87% at P-B reactor outlet, and it was 5–42% and 4–38% for the NA-BR and A-BR reactors, respectively. While the microbial community can degrade, on average, 40% of 2,4-D in 50 mg L^{-1} in 5760 min, a lag phase is observed for longer than 1440 min in both aerated and non-aerated reactors

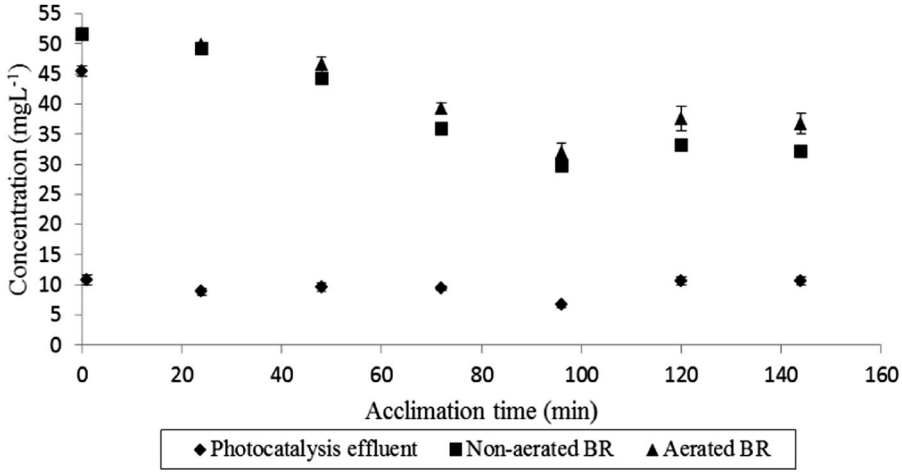


Figure 3. Time-dependent 2,4-D degradation by 3 different bioreactors (● photocatalysis effluent of 15 min in hybrid reactors (P&B), ■ non-aerated bioreactor (NA-BR), ▲aerated bioreactor (A-BR))

(Figure 3). However, in the photobiolysis reactor, it is seen that there is a phase transition from the lag phase to the log phase in less than 60 min, 79.12 ± 1.77% of herbicide removal is observed at the end of 60 min, and 87.14 ± 0.75% at the end of 5760 min (Figure 3). At the same time, all reactors could reach the stationary phase at the end of 5760 min. In the photocatalysis experiment, 2,4-D degradation around 55% was obtained from 25 to 11.43 mg L^{-1} after 15 min. Also, the initial 2,4-D concentration (50 mg L^{-1}) was decreased to 45 mg L^{-1} after 15 min of photocatalysis, and further decreased to 10.79 mg L^{-1} after 60 min with biological treatment. This means, when the initial pollutant concentration increased, apparent rate constant (k_{ap}) might decrease from 0.0441 min^{-1} at 25 mg L^{-1} to 0.0214 min^{-1} at 50 mg L^{-1}. In the study, the initial 2,4-D concentration (50 mg L^{-1}) decreased to 45 mg L^{-1} after 15 min of photocatalysis, and further decreased to 10.79 mg L^{-1} after 60 min with biological treatment. Similarly, according to a study by Yahiat et al. [16], the concentration decreased to 12 mg L^{-1} because of a photocatalysis process with 85 mg L^{-1} of cyproconazole fungicide, which in turn increased its biodegradability. In this study, a 50 mg L^{-1} concentration for A-BR and NA-BR at the end of 2880 min did not have a significant toxic effect on the microbial population of 2,4-D. The low amount of 2,4-D removal obtained for A-BR and NA-BR, using direct biological oxidation, reveals the necessity of pre-treatment with photocatalysis before applying the biological treatment. Similarly, Yahiat et al. [16] indicated that 85 mg L^{-1} of cyproconazole fungicide only started to be consumed at the end of 3600 min in an environment including both glucose and ammonium, and a significant amount of growth was recorded in the growth phase (log phase) at the end of 3600 min. This phenomenon revealed that ammonium and glucose provide a carbon and nitrogen source for growth. Consumption of the pollutant at the end of 3600 min confirmed that this substance was used as an energy source in the stationary growth phase. Accordingly, the observed growth showed that this concentration did not have a toxic effect on *P. fluorescens* cells. The microbial lag phase time was shortened with a photochemical pre-treatment process, and the amount of organic macro-pollutants having high biodegradability – which can be degraded by microorganisms – was decreased. The reason for this can be the oxidation of the benzene ring before its separation in the biodegradation mechanism of 2,4-D [8]. Furthermore, as a result of 15 min photocatalysis of a 50 mg L^{-1} concentration of 2,4-D, 5 mg L^{-1} of it transformed into easily degradable byproducts and thus the biomass of the community increased; the remaining 45 mg L^{-1} enabled the formation of a high amount of digestive enzyme that can accelerate the biodegradation of 2,4-D [8].

However, since the amount of electricity consumed during photocatalytic process was also important in terms of cost, it was of great importance to carry out the biological treatment by limiting the photocatalytic process time to a minimum. In this study, the total cost was 247.59 USD kg^{-1} (881.56 TRY kg^{-1}) in the biological treatment system, where a 15-min pre-photocatalytic treatment was applied, and then 50 mg L^{-1} of 2,4-D was removed after applying a 5760 min aerated process, while the total cost for the UV/TiO_2 photocatalysis process for 180 min – where a maximum amount of removal was observed for 50 mg L^{-1} of 2,4-D – was 723.86 USD kg^{-1} (2579.17 TRY kg^{-1}), indicating that the cost was approximately three times as much. With the hybrid system, 90% of the 2,4-D herbicide could be

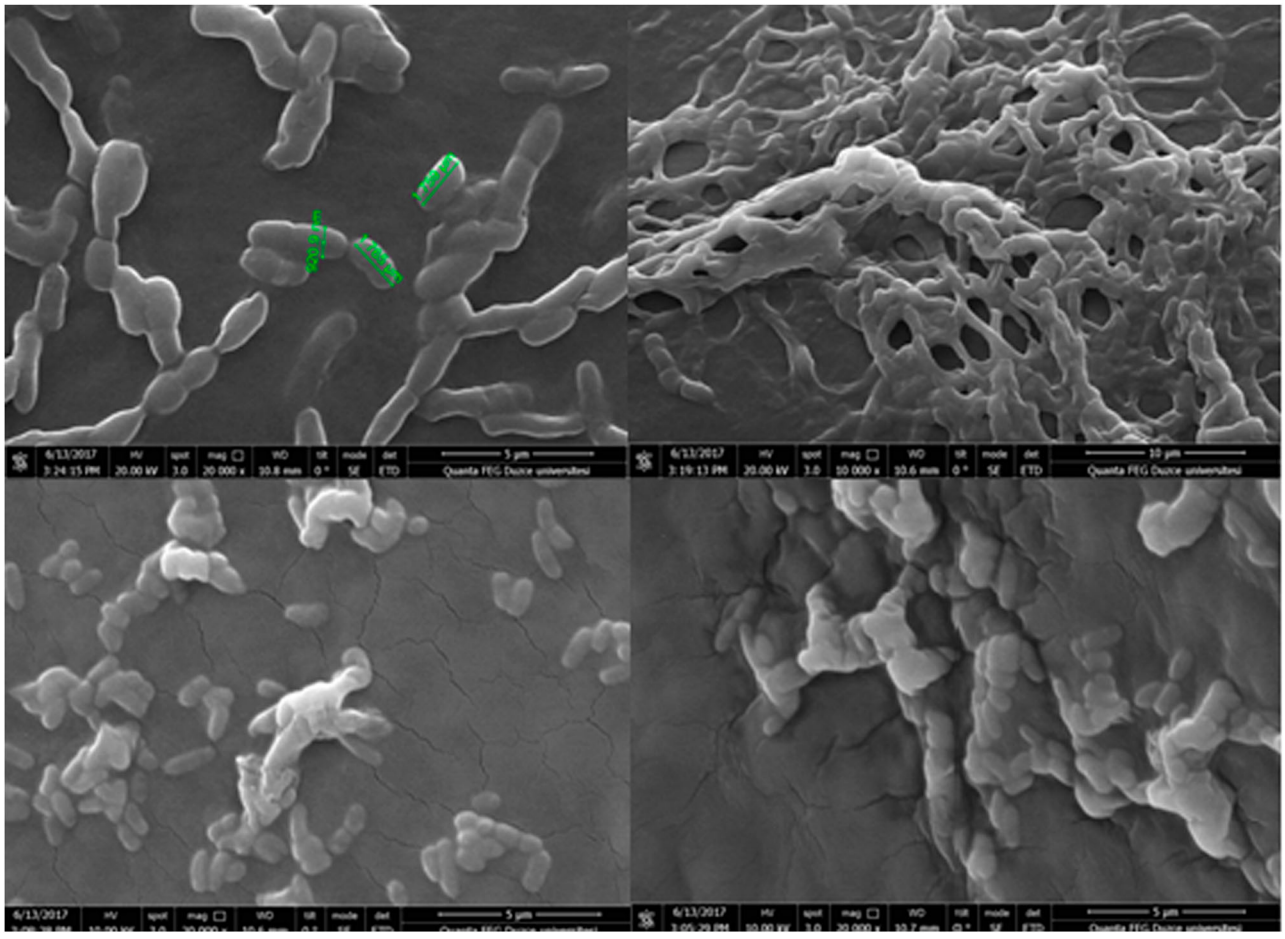


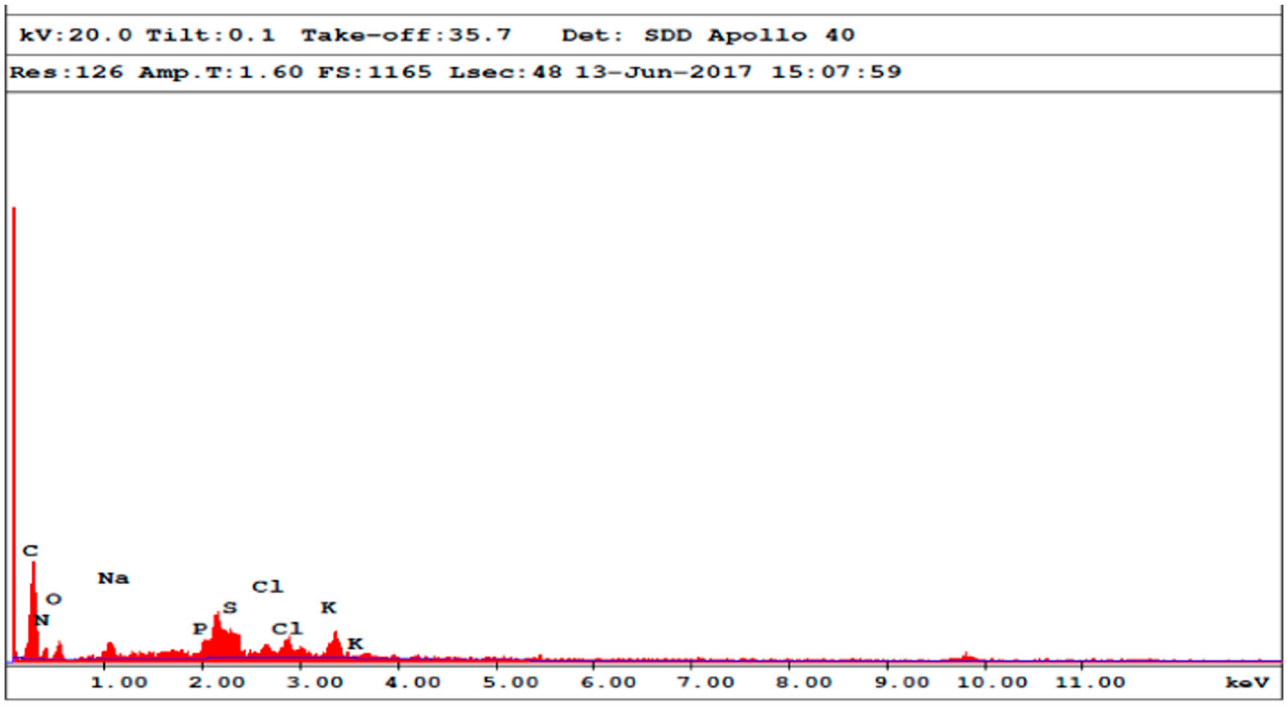
Figure 4. SEM image of rod biofilm bacteria obtained from biological reactors.

removed and organic pollutant removal was achieved at a lower cost in a shorter time than with the photocatalytic system.

Bioreactors, where immobilized cells are present, are usually the most efficient way to treat organic pollutants with biological treatment because biofilm processes are less sensitive to toxic substances than suspended processes [31]. Figure 4 shows the SEM image of the biofilm community while Figure 5 shows the elemental analysis of the microbial community performed by Energy Dispersive X-Ray Spectroscopy (EDS) analysis. Microorganism cells are composed of 50% (by weight) carbon (C), 14% nitrogen (N) and the remaining amount is sulphur (S), phosphorus (P) and trace elements (Ca, Mg, K, Na, Fe, Cu, Zn and etc.) [32]. According to the EDS analyses in Figure 5, the elemental analysis that indicates the presence of the microbial community was shown. Moreover, according to the EDS analysis, the elements of the 2,4-D salt ($C_{10}H_{13}Cl_2NO_3$) in the adsorption onto the TiO_2 catalyst are seen at the EDS peaks.

3.4. Biological treatment performance on 2,4-D mineralization

Evaluating chemical and biological treatment efficiencies, the maximum COD removal efficiency for 2,4-D reached 62.47 ± 0.70% after 210 min with 1.5 g L^{-1} of TiO_2 and UVA irradiation (Figure 6). When COD removal efficiencies in aerated and non-aerated biological reactors were examined, both reactors reached 78% after 5760 min, while in the aerated biological reactor, the COD removal efficiency was higher than that in the non-aerated reactor. Moreover, the COD removal was 67.75 ± 1.05% after 2880 min in the aerated reactor, while it was 49.71 ± 0.33% after 2880 min in the non-aerated biological reactor. Accordingly, it was verified that biological processes are slower than chemical processes and require longer times to reach the same removal efficiency as photocatalytic treatment [33]. Ballesteros-Martín et al. [9] reported that 31% of dissolved organic carbon (DOC) was removed in 140 min by the photo-Fenton reaction. Within 300 min, 90% DOC removal was achieved with



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	56.17	66.92	0.1628	1.0184	0.2845	1.0002
N K	14.43	14.74	0.0149	1.0095	0.1025	1.0002
O K	11.26	10.07	0.0158	1.0015	0.1401	1.0001
NaK	3.48	2.17	0.0155	0.9376	0.4744	1.0008
P K	2.77	1.28	0.0241	0.9221	0.9336	1.0090
S K	4.69	2.09	0.0423	0.9404	0.9527	1.0067
ClK	2.59	1.04	0.0222	0.9000	0.9475	1.0056
K K	4.62	1.69	0.0412	0.9082	0.9835	1.0000
Total	100.00	100.00				

Figure 5. EDX graph and elemental composition (%) of the biofilm.

the biologically active sludge process. A total of 200 mg L^{-1} dimethoate, oxydemethon-methyl, carbaryl and methidathion pesticide mixture was removed with the combined system within 420 min.

With the, a maximum of 88.87 ± 0.12% COD removal was obtained after 5760 min. In this study, with the P-B system, a maximum of 88.87 ± 0.12% COD removal was obtained after 5760 min (Figure 6). In addition, the total COD removal was 88.87% with the combined system; it was 18.31% for the first 15 min by photocatalytic treatment, and it was 70.56% after 5760 min (Figure 7). Fontmorin et al. [28] treated the commercial

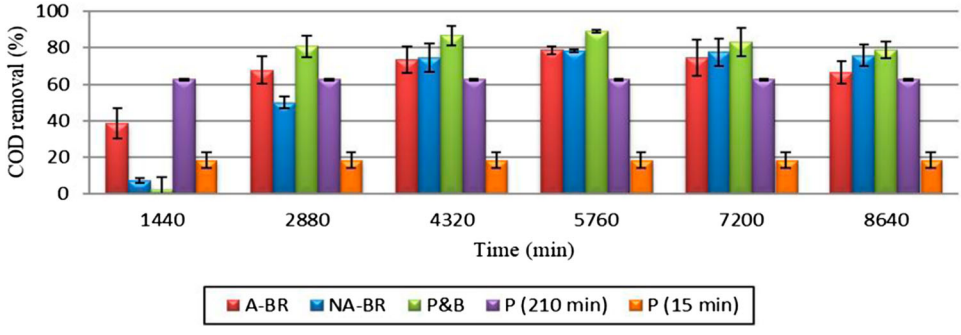


Figure 6. COD % removal for different biological reactors (A-BR: aerated biological reactor, NA-BR: non-aerated biological reactor, P&B: photobiocatalysis reactor, P (210 min): 210 min-photocatalysis, P (15 min): 15 min-photocatalysis.

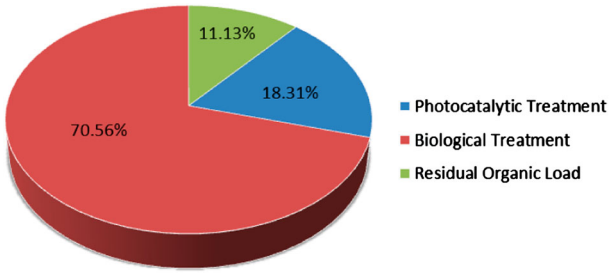


Figure 7. Mineralization efficiency obtained by combined system.

2,4-D herbicide (U46D®) by a combined electrochemical and activated sludge method, and the mineralization of U46D® (100 mg L^{-1} of 2,4-D) was limited without any pre-treatment after 6 days, while they obtained 33.7% DOC removal after 8 days. In the U46D® herbicide exposed to electrochemical pre-treatment, 63.7% DOC was observed up to 5 days, but it could not reach total mineralization (72.1%). With the combined system, 82.1% mineralization was obtained.

3.5. MALDI-TOF-MS identification in biological reactors

The aim was to determine if the dominant microbial species in the biological reactor, which fed the photoreactor output, was greater than other biological reactors, and determine whether microbial diversity was affected by the performance of the reactor [17]. In total, 13 isolates were identified by MALDI-TOF-TOF/MS, including four in the input activated sludge sample fed into the biological reactors, three in the sample obtained from the aerated biological reactor, two in the sample obtained from the NA-BR, and four in the biological reactant fed to the exposed water subjected to photocatalytic pre-treatment. Because of inoculation on PCA medium followed by a 2880 min incubation period, dominant colonies with different morphologies were obtained by passive colonization techniques and pure cultures were inoculated on separate petri dishes by an aseptic technique and reproduced after 1440 min incubation before being identified by the MALDI-TOF-TOF/MS device.

Bacillus subtilis, *Bacillus simplex*, *Ralstonia pickettii*, and *Acinetobacter towneri* bacteria isolates were detected with the highest accuracy in the samples obtained from the influent wastewater fed to all three biological reactors (Figure S1). It was reported by Kim et al. [34] *R. pickettii* (*Pseudomonas pickettii*) can degrade 2,4-D. Marrón-Montiel et al. [35] determined that, for the first time, *Acinetobacter* bacteria could degrade 2,4-D herbicide. Table 1 shows microorganism species identified according to reactor type, as well as their characteristics. As can be seen from the raw MALDI-TOF-TOF/MS profiles in Figure S1 to 4, densities and numbers of mass signals show differences. MALDI-TOF-TOF/MS profiles were compared with the reference spectrum in the BioTyper database and the similarities were identified by BioTyper Log (score). The colour panels are (A-M) rod spectra, they were matched with the experimental MALDI profiles of the strains (the colour panel on top), and with reference MALDI profiles (the blue panel at the bottom). Green, yellow and red rods on the colour panel indicate perfect, moderate and low peak match (density and *m/z* value) between experimental results and the reference MALDI database, respectively [18]. Matching pairs are evaluated by Log (score). At the same time, as a result of a comparison of the peaks obtained and the database, the first ten samples are rated from the highest accuracy (green) to the least accuracy (red) (Figures S1–S4). The Log (score) is calculated by the peak weight representing the correlation factor related to the number of matched peaks, total peak number, originality of species and the densities of matched peaks [36]. For *B. subtilis*, accurate identification at kind level could be made with the DT method, while accurate identification at species level

Table 1. Microorganism species identified in the biological reactors and their characteristics.

Biological reactor	Microorganism species	Microorganism characteristics
Biological reactor inlet water sample	*B. subtilis*	Gram (+), Basil, strict aerobe or facultative anaerobe
	R. pickettii	Gram (−), Basil, strict aerobe
	B. simplex	Gram (+), Basil, strict aerobe or facultative anaerobe
	A. towneri	Gram (−), Basil, strict aerobe
Aerated biological reactor sample	*S. acidaminiphila*	Gram (−), Basil, strict aerobe, belonging to the Xanthomonadaceae family and a Stenotrophomonas sub-species
	E. coli	Gram (−), Basil, Enterobacter, Facultative anaerobe
	R. ornithinolytica	Gram (−), aerobe, an Enterobacter species of the Klebsiella group
Non-aerated biological reactor sample	*K. pneumoniae*	Gram (−), anaerobe, a fermenting bacteria in the Enterobacter family
	C. freundii	Gram (−), Facultative anaerobe, in the Enterobacter family, Basil
Biological reactor sample subjected to photocatalytic pre-treatment	*B. subtilis*	Gram (+), Basil, strict aerobe or facultative anaerobe
	C. freundii	Gram (−), Facultative anaerobe, in the Enterobacter family, Basil
	A. eucrenophila	Gram (−), a kind of aeromonas, facultative anaerobe, Basil
	S. multivorum	Gram (−), a species of Flavobacterium, aerobe

could be made with the formic acid (FA) method. With the DT method, *R. pickettii* could be identified at species level. *B. simplex* and *A. towneri* could be identified at kind level, while a successful identification could not be made using the FA method.In the A-BR system, *Stenotrophomonas acidaminiphila*, *Escherichia coli*, *Raoultella ornithinolytica* bacteria were identified as dominant (Figure S2). Samir et al. [8] showed that *Stenotrophomonas maltophilia* species could biologically degrade the 2,4-D herbicide.

In the NA-BR, *Klebsiella pneumoniae* and *Citrobacter freundii* were identified as dominant. Marrón-Montiel et al. [35] determined that for the first time *Klebsiella* bacteria played a role in degrading 2,4-D herbicide.

In biological reactor samples subjected to photocatalysis pre-treatment, *B. subtilis*, *C. freundii*, *Aeromonas eucrenophila* and *Sphingobacterium multivorum* bacteria species were observed to be dominant (Figure S4, Table 1). Furthermore, it is known that *Sphingobacterium* species are among those that can degrade 2,4-D [37] (Figure S4). Liberatore et al. [14] identified *Sphingobacterium* in a bacterial community while removing Dazomet and Fenamiphos pesticides using a combined chemical and biological treatment, because of examining microflora in the biological treatment. Kim et al. [38] reported that *Aeromonas sp.* species was used in the biodegradation of 2,4-D herbicide. Sandoval-Carrasco et al. [39] identified five different bacteria isolates in the removal of 2,4-D herbicide: *Aeromonas*, *Acidovorax*, *Chryseobacterium*, *Variovorax* and *Xanthobacter*.

The 2,4-D is firstly degraded by *Achromobacter*, *Bordetella*, *Xanthobacter*, *Streptomyces*, *Aspergillus*, *Corynebacterium*, *Nocardia*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium* and *Pseudomonas*. The members of the last four groups use the 2,4-D as a single carbon source [40]. Accordingly, in this study, the *A. eucrenophila*, *S. acidaminiphila*, *R. pickettii*, *S. multivorum* and *A. towneri* species identified with high accuracy played important roles in the degradation of 2,4-D.

According to the results, although the microbial species developed in each reactor differ from each other, *C. freundii* was not observed to be dominant in the aerated reactor and influent, but it was seen in the biological treatment of the wastewater subjected to photocatalytic pre-treatment by a non-aerated biological reactor. *B. subtilis* was observed in both influent samples and the biological treatment system of the wastewater subjected to photocatalytic pre-treatment.The observation of different bacterial species in three different reactor systems can be attributed to the reactor characteristics of microbial ecologies in bioreactors [21].

According to Goel et al. [16], the addition of glucose is important to achieve complete mineralization of toxic compounds. Similar results obtained from the combined system of MCP at different glucose concentrations (0, 1 and 2 g L^{-1}) after 3600 min show completed TOC removal, and the addition of a biogenic substrate accelerated the growth of microorganisms. Accordingly, in this study after 4 days, it is thought that microbial growth was inhibited by biologically toxic substances formed due to the degradation of the 2,4-D and the herbicide mineralization and degradation decreased after 5760 min (depending on microbial growth inhibition) before becoming stable. As a result, it is predicted that after 5760 min, mineralization can increase to 90% by improving the microbial growth with the addition of a glucose substrate.

4. Conclusions

The combination of photocatalytic and biological oxidation was demonstrated to be an effective treatment for rapid herbicide degradation (80.89 ± 0.81% COD and 81.36 ± 1.37% 2,4-D removal were achieved after 2 days) in water containing 50 mg L^{-1} of herbicide. However, the COD removal was 67.75 ± 1.05% after 2 days in the aerated reactor, while it was 49.71 ± 0.33% after 48 h in the non-aerated biological reactor. This means, biodegradation alone did not mineralize the COD by more than 68%. Thus, the concept and benefit of coupling photocatalysis and biodegradation were demonstrated in this study. According to MALDI-TOF-TOF/MS results, the microbial species developed in each reactor show differences. Furthermore, that various bacterial species were observed in three different reactors may be due to the reactor characteristics of microbial ecologies in bioreactors.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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