Desalination and Water Treatment 322 (2025) 101129

Contents lists available at ScienceDirect

Desalination and Water Treatment

journal homepage: www.sciencedirect.com/journal/desalination-and-water-treatment/

Integrating response surface methodology and decision tree algorithms for valorization of cheese whey wastewater

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ARTICLE INFO

Keywords:
Cheese whey wastewater
Microalgae
Nannochloropsis sp.
Optimization
Response surface analysis
Decision tree
Machine learning

ABSTRACT

Recently, the potential of microalgae in wastewater treatment has attracted attention. The goal of this study is to find optimum conditions for microalgae growth and the concentration of cheese whey wastewater (CWW) to get the best treatment efficiency by using response surface methodology (RSM) and the decision tree algorithm for different pollutant parameters. The study used reactors with different amounts of CWW and *Nannochloropsis sp.* to find the best concentrations for each parameter. The best concentration of CWW was found to be 8000 mgCOD/L, and the best concentration of *Nannochloropsis* sp. microalgae was found to be 2200 mgVS/L. It was found that Chemical Oxygen Demand (COD), Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), and Orthophosphorus (Ortho-P) could be removed at different ranges, 77–96 %, 95–98 %, 51–97 %, and 60–99 % of CWW, respectively, depending on the different combinations of microalgae and CWW concentrations. The desirability values in RSM for COD, TOC, TKN, and Ortho-P parameters to be 0.99, 0.94, 0.78, and 0.63, respectively. The study suggests the marine microalgae (*Nannochloropsis sp.)* could be an alternative way to treat saline CWW, to create a circular economy. The machine learning (ML) method validates that RSM predictions are consistent and accurate. The results show that it is possible to combine traditional optimization methods with more advanced ML methods to facilitate the design and the operation of the treatment plants.

1. Introduction

Population increase and the growth of industrial activities brought about waste and wastewater production, resulting in detrimental changes in the ecosystem including climate crisis and water scarcity [1]. Traditionally, wastewater treatment has been a primary focus for protecting the environment [2]. However, this paradigm has changed to the development of the circular economy that enables the recovery of resources with economic value from the wastewater or generating new materials for diverse applications [3].

The dairy sector, especially cheese production, is one of the most prominent producers of industrial wastewater in the world [4]. Türkiye produced about 69 thousand 516 tons of cheese products in July 2023, as reported by the Turkish Statistical Institute [5], 2023 and approximately 20 % of this production generates cheese whey wastewater (CWW). In rural areas, the wastewaters are generally not properly treated and cause serious environmental problems [6]. CWWs, which mainly consist of carbohydrates, lactose, and oil, are characterized by their high organic load and salt content [7]. The application of the conventional treatment processes to the wastewater is associated with several disadvantages such as high energy input, large land requirements, high operating costs and the need for advanced treatment processes for nutrient removal from CWW [8,9]. For this reason, alternative options should be investigated for the management of the wastewater. In recent years, there has been a growing interest in conducting studies for the conversion of pollutants in wastewater into more valuable or alternative products [10]. For the management of the CWW, utilizing microalgae can hold a promising alternative in both treatment of the pollutants and production of cost-effective products [11]. In terms of complex wastewaters such as CWW, this approach will contribute to the circular economy and represent a milestone for attaining sustainable development goals [12].

Microalgae, which have enormous biodiversity, require only light and nutrients, chiefly nitrogen and phosphorus, for growth. However, it has been stated that mass production and commercialization of microalgae have been limited by the challenges associated with the cost of growing, harvesting, and processing algal biomass [13]. As many researchers have also pointed out, microalgae production is an expensive

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https://doi.org/10.1016/j.dwt.2025.101129

Available online 20 March 2025

process due to the essential nutrients required to sustain the system [14]. Nutrients, which are one of the main costs for cultivating microalgae, are found in abundance in the several wastewaters. Therefore, it is a viable alternative to use specific wastewaters that naturally contain the nutrients to produce microalgae [15] such as CWW. However, it is of paramount importance to optimize the microalgae-based systems in terms of the both microalgae and influent wastewater concentration to operate the system effectively and maximize the performance. Microalgae technology is considered a process that is known to be especially effective in nutrient removal in conventional wastewater treatment plants [16]. *Nannochloropsis sp.,* which is a marine and mixotrophic microalgae [17], has a potential for both carbon and nutrient removal and it can be a viable candidate for saline wastewaters.

Nannochloropsis sp. is characterized by its high biomass productivity, rapid growth rate, and high lipid yield [18]. This biochemical profile enhances the commercial potential of *Nannochloropsis sp.* in the nutraceutical and animal feed sectors beyond biofuel applications [19]. Additionally, its wide environmental tolerance and adaptation to high salinity levels allow for low-cost cultivation while enabling it to be produced in open systems without competing with freshwater resources [20]. It has been reported that *Nannochloropsis sp.*, which can adapt well to natural seawater conditions, can grow at salinity levels of up to 50 ppt and continue lipid production [21]. However, it has been noted that higher salinity levels may negatively affect the enzymatic activities necessary for lipid biosynthesis [22]. This characteristic, especially in the treatment of saline industrial wastewater, enables the availability of *Nannochloropsis sp.* and offers opportunities for new approaches that integrate biofuel production with environmental sustainability. *Nannochloropsis sp.* has been tested for municipal wastewater [23] as well as industrial wastewaters such as pulp and paper [24], pharmaceutical [25], and palm oil mill [26]. Moreover, it has potential use in various applications, such as biofuel [27] and biogas production [28]. The choice of *Nannochloropsis sp.* for the study was motivated by its ability to thrive in saline environments, making it a suitable candidate for treating CWW, which is a highly saline and problematic wastewater. Additionally, previous research has shown limited investigation of *Nannochloropsis sp.* in wastewater treatment, especially in the context of treating CWW. Thus, this study fills a gap in the literature by exploring the potential of *Nannochloropsis sp.* for CWW treatment. Utilizing CWW as a nutrient source for microorganisms will not only contribute to reducing pollutant levels in the wastewater but also allow for the utilization of grown microalgae as an energy material and valuable products in various processes. However, microalgae require certain growth conditions, such as temperature, light intensity, pH, inorganic carbon source, nutrient levels, and mixing in order to grow and effectively remove nutrients from wastewater [29]. Therefore, it is essential to adjust the process and environmental conditions when using *Nannochloropsis sp.* for the effective removal of pollutants from CWW. The treatment efficiency of wastewater can be increased by choosing many parameters affecting the process in the appropriate range. It is critical to optimize the operational conditions for microalgae in wastewater treatment studies. The content of industrial wastewater is diverse, so optimization is reasonably important for these wastewaters. In traditional optimization studies, a variable is held constant, but the relationship between variables cannot be properly explained with the methods [30]. Response Surface Methodology (RSM), which is a statistical method, can be used to design experiments, build models, evaluate factors, and find optimal conditions for desirable responses [31]. RSM optimizes the several parameters affecting the processes and the growth conditions for microalgae, which can provide higher treatment yields and lower operating expenses. It is also effective in optimizing experimental procedures and reducing chemical consumption [32]. In the literature, there are studies with microalgae using RSM for municipal wastewater [33], palm oil mill effluent [34], mining tailings [35]. RSM can be used to create a mathematical model that relates microalgae performance to various factors such as light period [34–36], light intensity [34], temperature [36,37], pH [36,37], microalgae concentration [38] and nutrient concentration [16,35] in the wastewater. This model can predict the optimal conditions for the best microalgae performance. However, RSM has shortcomings in its ability to describe non-linear model behavior, so ML algorithms can be used to describe input and output relationships in the treatment processes [39]. ML can be used in wastewater treatment systems for real-time monitoring, optimization, uncertainty prediction, and defect detection [40,41]. Utilizing the advantages of both statistical and machine learning methodologies facilitates a more thorough comprehension of the system, hence enhancing wastewater treatment operations [42,43]. In this study, RSM was preferred to explain the interaction between variables in determining the optimum operating conditions, and in addition, the machine learning algorithm decision tree was preferred to test the accuracy of the RSM results. The incorporation of decision trees into optimization research enhances conventional techniques such as RSM, providing a data-driven approach that can yield more accurate and dependable results [44]. For optimizing treatment microalgae concentration and CWW concentration were identified as important parameters.

The hybrid integration of RSM and ML has demonstrated potential in enhancing the predictive accuracy of optimization models for biomass growth and wastewater treatment efficiency. Despite the increasing interest in RSM-ML integration, studies remain limited. Notable examples include the application of RSM and Artificial Neural Networks (ANNs) to optimize biodiesel production from *Chlorella pyrenoidosa*, reducing experimental trial-and-error costs and improving predictive accuracy [45]. Similarly, RSM, Analysis of Variance (ANOVA), and Deep Neural Networks have been utilized to optimize photobioreactor conditions for bioremediation using *Chlorella vulgaris* [46]. Additionally, AdaBoost and XGBoost algorithms combined with RSM have been employed to assess microalgae's impact on organic matter and nutrient concentrations in greywater [47]. However, the existing literature lacks studies focusing on the optimization of microalgae-based wastewater treatment through the combined use of RSM and ML algorithms, highlighting a gap for future research.

In this context, the study aimed to investigate the possibility of treating CWW by using *Nannochloropsis sp.* (a marine microalgae) and simultaneously producing microalgae which have many different application areas in the field. For this purpose, RSM was used to optimize both microalgae and CWW concentration. The decision tree, a machine learning technique, was employed to accurately assess the optimization results and to reduce potential deviations inside the system. In addition, wastewater characteristics, absorbance results (at 680 nm), and chlorophyll-a parameters, a good biological indicator in the water body, were monitored during the study. This research provides a reference for an alternative treatment method for CWW which is one of the most polluting industrial wastewater by marine microalgae for achieving a circular economy based on microalgae growth which has many potential usage areas. This study addresses these gaps by combining advanced optimization methods with the microalgae-based valorization of CWW. This study not only contributes to "Clean Water and Sanitation (SDG6)" but also highlights "Sustainable Cities and Communities (SDG11)" by addressing complex industrial wastewater situations. Furthermore, the valorization of biomass is consistent with "Responsible Consumption and Production (SDG12)," emphasizing the work's broader environmental and economic consequences.

2. Materials and methods

In this study, appropriate concentration ranges for *Nannochloropsis sp.* microalgae were first determined for different concentrations of the CWW using Minitab program. Under these determined conditions, the reactors were operated, and the removal efficiencies, particularly in terms of Chemical Oxygen Demand (COD), Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), and Orthophosphorus (Ortho-P), were determined. Then, decision tree and RSM analyses were conducted, and the performance of these analyses in determining concentrations based on pollutant parameter removal efficiencies was both evaluated and compared.

2.1. Microalgae

Nannochloropsis sp. was obtained from a fish farm in Çanakkale. The microalgae biomass was grown to the log growth stage [48] and used for experiments. *Nannochloropsis sp.* were cultured in pure water enriched with 100 g/L $MgSO_4$, 100 g/L $MgCl_2$, 150 g/L $CaCl_2$, 100 g/L KNO_3, 4 g/L $NaHCO_3$ and 7 g/L KH_2PO_4. The experimental setup consisted of a bioreactor open to the atmosphere with a total working volume of 2 L. Microalgae incubation was carried out at pH 8–8.2 for about 15 days. Salinity was kept constant in the range of 35–37 ‰. Microalgae growth was determined by spectrophotometry at OD (optical density) 680 nm.

2.2. Cheese whey wastewater

In the experiments, CWW was used to grow microalgal biomass under optimal conditions. We collected CWW samples from a cheese production facility located in the Ezine district of Çanakkale province. CWW has 1410 mg/L of TKN, 442 mg/L of Ortho-P, 19994 mg/L of Cl-, 74820 mg/L of COD, and 24975 mg/L of TOC. It also has 63580 mg/L of total solids and 57880 mg/L of volatile solids. These given values are valid for raw CWW. The COD concentration of raw CWW generally ranges from 50 to 80 g/L, depending on the source and processing conditions. However, our CWW had significantly high COD levels compared to the literature. Based on preliminary studies, we adjusted the COD concentration to 8000 mg/L to discover the operational conditions of the reactor. The concentration of other parameters, including TKN, Ortho-P, TOC and solids content, decreased due to the dilution by a factor of about 10. Detailed information related to the preliminary studies was also stated in the Results and Discussion Section (*3.1*. *'Preliminary Studies with Cheese Whey Wastewater'*).

2.3. Experimental set-up used for cheese whey wastewater optimization

Glass batch reactors with an active volume of 250 ML were used in this study. The reactors were operated open to the atmosphere. Operating conditions were designed based on the data obtained from Minitab.

In this study, CWW concentrations were tested between 1000 mgCOD/L and 8000 mgCOD/L concentrations while microalgae concentration was used between 1000 mgVS/L and 8000 mgVS/L. In this study, CWW concentration ranges were chosen as 1000–8000 mgCOD/L based on preliminary experiments. However, a starting microalgae concentration was chosen as 1000 mgVS/L from the literature. The maximum VS concentration (8000 mgVS/L) was obtained from experimental studies in the laboratory.

The concentration ranges of microalgae used in wastewater treatment can vary depending on various factors, such as the type of wastewater, specific microalgae species, and the operational parameters of the treatment process. Wastewater treatment with *Scenedesmus sp.* [49] and *Desmodesmus communis* [50] microalgae strains, concentrations between 50 mg/L and 1 g/L, have been shown to be beneficial, especially in controlled environments such as photobioreactors and batch reactors. In an initial study, it was determined that maintaining *Scenedesmus sp.* biomass concentrations around 1.2 g/L could be a potential concentration range for nutrient removal and biomass production [51]. Therefore, a starting microalgae concentration was chosen as 1000 mgVS/L from the literature in this study. However, the optimum concentration of microalgae depends on the characteristics of the wastewater being treated, and evaluating it based on a single value can be misleading. Therefore, different VS concentrations were tested, and the maximum VS concentration (8000 mgVS/L) was obtained from experimental studies in the laboratory.

The process with microalgae and CWW was carried out for four weeks with an average light of 5000 lux, 170 rpm mixing, 24:0 (light: dark) photoperiod, room temperature and 15 days incubation period. In the literature, optimization processes have been carried out in studies where microalgae are cultivated with wastewater, taking into account various parameters. Among these parameters, light intensity, photoperiod, temperature, and nutrient concentrations play important roles. For light intensity, ranges between 1000 and 8000 lux are generally examined, and in the majority of studies, it has been reported that approximately 5000 lux levels have positive effects on microalgae growth [52–54]. In this context, the light intensity in the study was set at 5000 lux, and this choice was made based on previous research in the literature and preliminary tests conducted in the laboratory environment. Additionally, the effects of different light/dark cycles (e.g., 6/18, 12/12, and continuous lighting 24:0) on microalgae growth have been examined in the literature, and it has been reported that high photoperiods increase microalgae growth [52,53]. Therefore, in the study, a 24-hour continuous illumination (24:0) photoperiod was preferred to achieve the highest level of microalgae growth.

The COD, TOC, TKN and Ortho-P parameters were analyzed for the samples taken at the beginning and at the end of the process. The removal efficiencies were calculated for each reactor. During the experiments, microalgal growth was monitored by using spectrophotometric analyses. In the reactors, carbon removal was monitored by COD and TOC parameters while nutrient removal was monitored by TKN and Ortho-P parameters.

2.4. Analytical methods

COD analyses were determined by a closed reflux method with DR 5000 spectrophotometer. Total suspended solids and total dissolved solids contents of the samples were determined by filtration (0.45 µm filter paper) and oven. Similarly, total solids and volatile solids contents were carried out by hot plate, oven (T = 105–107 °C for 1 hour) and muffle furnace (T = 550–600 °C for 30 minutes). TOC was determined with the HACH IL550 model TOC/TN. The combustion unit and distillation unit were used for TKN determination. Ortho-P was determined by the DR 5000 spectrophotometer. The optical density of the microalgae culture suspension was measured at 680 nm using a DR 5000 spectrophotometer.

Chlorophyll-a analysis was conducted to examine the photosynthetic activities and growth of microalgae. The analysis was carried out as described in Toyub et al. [55]. A volume of 10 ML was taken from each sample. Centrifugation was performed at 3000 rpm for 1 minute, and the supernatant was separated. Acetone was added to the remaining solid fraction. The samples were analyzed using a spectrophotometer at 664 nm, 647 nm, and 630 nm. The obtained results were inserted into Eq. 1 provided below, and the final results were obtained in mg/L [55].

$$\text{Chlorophyll-a (mg/L)} = 11.85\,(OD\ 664) - 1.54\,(OD\ 647) - 0.08\,(OD\ 630) \quad (1)$$

2.5. Statistical analysis

RSM is used to systematically investigate and optimize the relationship between a large number of input variables and their corresponding output responses. RSM can identify the optimal combination of factors required to achieve the most desirable outcome [56]. Statistical analyses of the data in this study were performed using Minitab (Version 20.4) software. In order to increase the pollution removal efficiency and biomass production capacity, the concentration factor was optimized using a Central Composite Design based on RSM. Optimization tables were used to determine the concentration values that give the best operational condition in terms of both pollution removal and biomass growth. Table 1 provides operational details of the 13 reactors used in the study, which contained a combination of CWW and microalgae (*Nannochloropsis sp.*) concentration (MA) according to the Minitab.

The Decision Tree method was carried out with RapidMiner Studio version 10.3 for the data modeling procedure. The decision tree flowchart is shown in Fig. 1. The carbon and nutrient removal efficiency of *Nannochloropsis sp.* in CWW was assessed using COD, TOC, TKN, and Ortho-P parameters, and a decision tree model was developed incorporating variables such as CWW concentration and *Nannochloropsis sp.* microalgae concentration. This model was employed for data classification and analysis.

3. Results and discussion

3.1. Preliminary studies with cheese whey wastewater

After the enrichment of the microalgae *Nannochloropsis sp.*, initial studies were conducted using various concentrations of CWW to determine the impact of the wastewater concentration on microalgae growth. The microalgae *Nannochloropsis sp.* was fed with different concentrations of CWW ranging from 1000 mgCOD/L to 20000 mgCOD/L. During the studies, microalgae concentration was kept constant at approximately 2600 mgVS/L at the beginning of the reactor studies.

In the present study, the effects of various CWW concentrations on the treatment process have been examined. Fig. 2 shows the biomass growth of *Nannochloropsis sp.* as a function of CWW concentration. Absorbance measurements at 680 nm were recorded for four days. As shown in Fig. 2, microalgae growth was negatively affected by CWW concentrations exceeding 8000 mgCOD/L. As a result, CWW concentrations greater than 8000 mgCOD/L were excluded from subsequent experiments. In this study, CWW concentrations ranges were chosen as between 1000 mgCOD/L and 8000 mgCOD/L based on preliminary experiments.

CWW is industrially sourced wastewater with high organic pollutant concentrations, containing various contaminants from industry processes [7]. In our study, we used a CWW with very high organic pollutant strength compared to literature. It has been highlighted that treatment of the high-strength industrial wastewaters with microalgae-based treatment processes requires dilution of the wastewater [57,58]. Direct use of the raw industrial wastewaters with a COD of > 70000 mg/L in the processes affects microalgae growth and nutrient removal due to the block of the light penetration caused by the color of the influent [59] or suspended solids and turbidity [60]. Therefore, in our study, the highest CWW COD concentration value that microalgae can tolerate (8000 mgCOD/L) was used. Due to the strength of the wastewater, we arranged wastewater strength by dilution in order to discover the maximum applied organic load of the system in our study. Clearly, this study can be applied to the other industrial wastewater or dairy industry wastewater with lower COD values (<8000 mg/L).

Studies in the literature have treated wastewater from the dairy industry using various microalgae species. Table 2 summarizes previous studies related to the performance of various microalgae in dairy wastewater treatment and applications of the microalgae species in different processes. The table shows that CWW has been used as feed for various microalgae species such as *Chlorella vulgaris* [61] *Oscillatoria sp.* [62] and *Scenedesmus obliquus* [63]. When the other wastewater studies are examined [64–66], it is generally observed that the removal

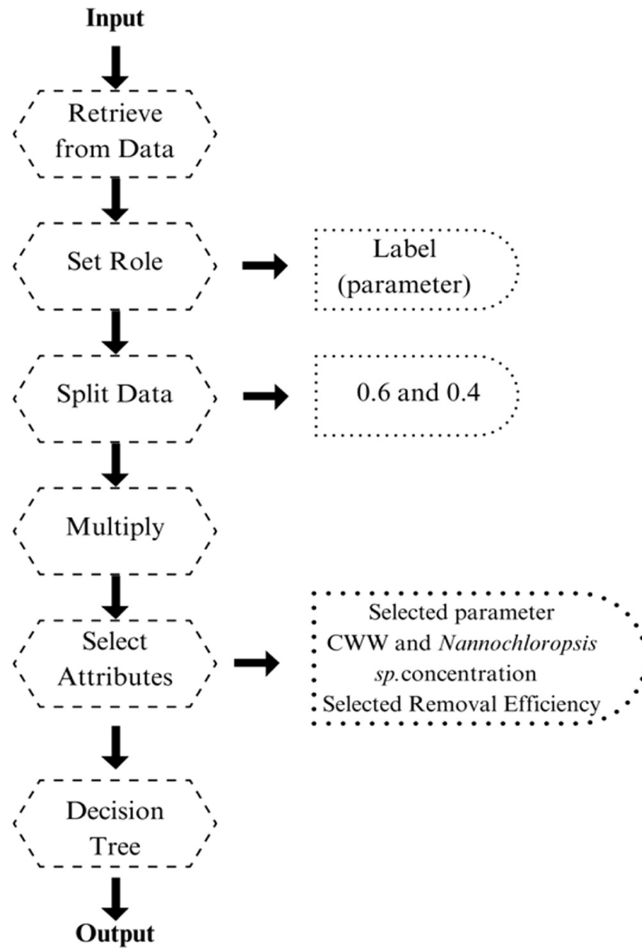

Fig. 1. Decision Tree flowchart.

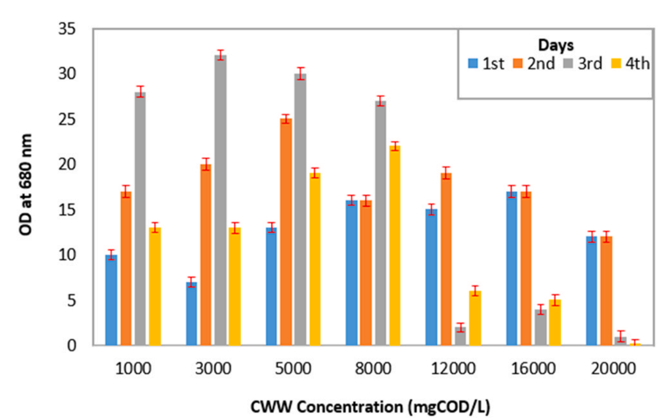

Fig. 2. The growth of *Nannochloropsis sp.* biomass depending on the CWW concentration.

Table 1
Experimental design proposed by Minitab.

No	CWW concentration (mgCOD/L)	Nannochloropsis sp. concentration (MA) (mgVS/L)
1	4500	4500
2	6975	2025
3	2025	2025
4	2025	6975
5	4500	4500
6	4500	1000
7	4500	4500
8	6975	6975
9	8000	4500
10	1000	4500
11	4500	4500
12	4500	8000
13	4500	4500

Table 2
Performance of various microalgae in dairy wastewater treatment and applications of the microalgae species in different processes.

Type of Microalgae	Wastewater	Goals and Results	References
AuxenoChlorella protothecoides	Dairy (COD: 3090 mg/L)	Removal efficiency COD:65 % TN:43 % P:77 %	[70]
Chlorella vulgaris	Dairy (COD:48 mg/L)	Removal efficiency TN:95 % TP:93 %	[64]
Arthrospira platensis	Dairy (COD: 119.21–140.17 ppm)	Removal efficiency COD:98 % N:99 % P:100 %	[65]
Chlorella vulgaris	Dairy (COD: 2452.3 ± 361.2 mg/L)	Removal efficiency COD:81 % N:85.5 % P:66 %	[66]
Chlorella vulgaris	Cheese whey (COD:42.47 ± 1.56 g/L)	Waste production Results C: 7–56 % N: 0–71 % P: 11–46 %	[61]
Nannochloropsis sp.	Municipal (after reverse osmosis) (COD:164 ± 7.8 mg/L, TN:43.2 ± 2.2 mg/L, TP:13.1 ± 0.4 mg/L)	Removal efficiency Results TN:> 50 % TP:> 80 %	[23]
Nannochloropsis sp.	Palm oil factory effluent (COD: 4196.67 mg/L, N:276.67 mg/L, P:50.44 mg/L)	Removal efficiency Results COD:48–55 % N:46–60 % P:44–62 %	[26]
Nannochloropsis oculata	Pulp and paper	Eicosapentaenoic acid production	[24]
Nannochloropsis sp.	Pharmaceutical effluent	Adsorption of heavy metals	[25]
Oscillatoria sp.	Cheese whey	Biobutanol production	[62]
Scenedesmus obliquus	Cheese whey	Biodiesel production	[63]

efficiencies of COD, TN, and TP are high. However, the influent wastewater COD concentrations used are usually very low compared to our study and real wastewater characteristics. The dairy industry and its sub-sector, CWW, generally contain a high organic load [67]. Therefore, despite the promising removal efficiencies, these results cannot reflect real industrial conditions, which have high organic content. In a study conducted using *AuxenoChlorella protothecoides*, it was observed that the removal efficiency was lower compared to other microalgae studies. This situation is related to the use of a much higher influent COD concentration. Therefore, in our study, concentration ranges were examined in detail for *Nannochloropsis sp.* microalgae at various operating conditions.

Several studies also stated that both microalgal growth and wastewater treatment efficiency are affected by the dilution ratio of the wastewaters [57] in microalgae-based systems. It has been stated that optimized microalgae growth parameters affect biomass concentration increase in microalgae-based processes [68]. Porto et al. [69] investigated the inhibitory effects of the secondary treatment of paper wastewater effluent at different dilutions on microalgal growth and nutrients with microalga *Chlorella vulgaris* and searched for satisfactory conditions. However, the studies do not include optimization with experimental design programs. Since microalgae concentration has paramount importance for the microalgal systems, it is critical to determine both CWW concentration and microalgae (*Nannochloropsis sp.*) concentration. For this purpose, optimization studies were carried out using Minitab in this study.

The use of microalgae in the treatment of wastewater from the dairy industry has not been sufficiently researched for large-scale applications. The scaling of this technology involves operational and economic challenges. Gramegna *et al.* [70] demonstrated that the applicability of the *AuxenoChlorella protothecoides* strain could be used in dairy industry wastewater through low-cost processes and nutrient optimization. Similarly, Choi [66] developed an efficient and economical treatment process with minimal chemical use. However, the installation costs of bioreactors and the need for continuous monitoring can create significant financial burdens. In terms of nutrient management, while high nitrogen and phosphorus content supports microalgae growth, excessive nutrient load can negatively affect lipid production and reduce biodiesel yield [71]. Therefore, the conditions such as strain selection, optimization of nutrient balance, and careful evaluation of scaling strategies are necessary to ensure operational sustainability [72]. For the sustainability of microalgae-based systems and their integration into real-scale wastewater treatment applications, it is particularly critical for saline industrial wastewaters to identify species that can provide both carbon and nitrogen removal, are salt-tolerant, and possess a wide environmental tolerance or adaptation capacity. Additionally, their suitability for low-cost cultivation and their ability to be produced in open systems without competing for freshwater resources are key factors that enhance the industrial-scale applicability of these species. Although microalgae such as *Nannochloropsis sp.* possess these characteristics, it is necessary to identify and test alternative species with similar traits. Therefore, expanding research on the large-scale use of microalgae-based wastewater treatment systems is of great importance.

3.2. Optimization with response surface methodology

As mentioned previously, it is crucial to optimize microalgae-based systems in terms of both the concentration of the microalgae in the reactor and influent wastewater concentration to operate the system efficiently and achieve maximum performance. Therefore, after the initial studies were completed, various concentrations of microalgae *Nannochloropsis sp.* were loaded into the reactors and fed with different concentrations of CWW according to the experimental design prepared with Minitab. Operational details of the 13 reactors used in the study were presented in Table 1 in the previous section. According to the experimental design proposed by Minitab, the reactors were operated with different combinations of CWW and *Nannochloropsis sp.* concentration. The pollutant parameters were characterized during reactor operation. The reactors were operated for approximately one month. In the reactors, carbon removal was monitored by COD and TOC parameters while nutrient removal was monitored by TKN and Ortho-P parameters. Fig. 3.(a,b,c,d) shows removal efficiencies of the reactors for the different pollutant parameters. When the graphs are examined, it can be seen that the carbon removal efficiency in the reactors is quite high owing to the mixotrophic characteristics of the microalgae.

Experiments were conducted using both sterilized and non-sterilized CWW to evaluate the possible impact of bacterial contamination in the microalgae-based treatment system. The growth of *Nannochloropsis sp.* and the overall effectiveness of the system were seen in both cases. No substantial differences were seen in growth patterns or pollutant removal efficacy between the sterilized and non-sterilized configurations. The results demonstrated that bacterial contamination did not substantially affect system performance under the examined conditions. Therefore, these findings were omitted from the study's main focus.

In Figs. 4, 3D response surfaces and contour plots are presented to investigate the effectiveness of *Nannochloropsis sp.* microalgae in CWW treatment under various influent concentration values. Based on the evaluation of the figures, depending on the different combinations of microalgae and CWW concentrations, the removal efficiencies of COD, TOC, TKN, and Ortho-P were found to be within the ranges of 77–96 %, 95–98 %, 51–97 %, and 60–99 %, respectively. COD removal is directly proportional to CWW concentration within the previously determined ranges. In reactors with CWW/MA ratio of 0.29, 0.56, 0.22, COD

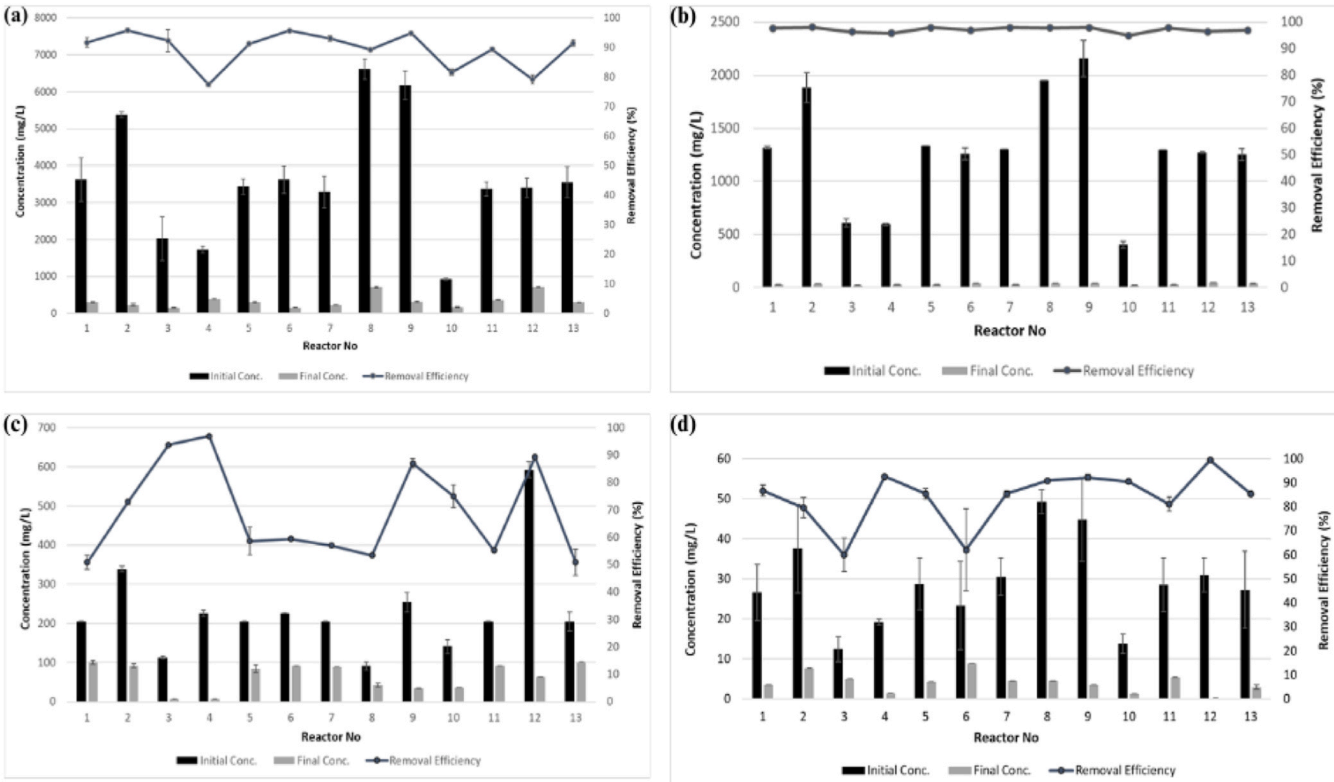

Fig. 3. The data presented include (a) COD; (b) TOC; (c) TKN; and (d) Ortho-P curves versus time and removal efficiencies of CWW and *Nannochloropsis sp.* at various reactors.

removal was 77 %, 79 %, 82 %, respectively. In reactors with CWW/MA ratio of 4.5 and 3.4, COD removal efficiency was found to be 96 % for both reactors. As long as COD does not exceed maximum limits, COD removal increases as CWW concentration increases. Xia and Murphy [73] stated that mixotrophic microalgae can play a role in the removal of approximately 50–90 % of COD. In our study, 51–91 % COD removal efficiency was also obtained by *Nannochloropsis sp.* which is a mixotrophic microalgae depending on the influent wastewater strength. In another study, De Almeida Pires et al. [61] studied the treatability of CWW by *C. vulgaris* microalgae with different percentages (1, 10, 25, 50, 100 %) and reported that the COD removal efficiency depends on the percentage of the wastewater. The efficiency has been found to be increased with increasing influent COD concentrations [61]. The microalgae is also a mixotrophic microalgae which can utilize both organic carbon in wastewater and CO_2.

TOC removal was found to be higher than 95 % in all reactors. In another study carried out with domestic wastewater using mixotrophic cultivation of *Chlorella vulgaris*, the removal efficiencies for COD, NH_4^+–N, and TP were found to fall within the ranges of 70–83 %, 51–91 %, and 30–94 %, respectively [16].

Nannochloropsis sp. is effective in wastewater treatment, removing nitrogen and phosphorus through various biological mechanisms including nutrient uptake, metabolic adaptations, and cellular regulatory mechanisms. *Nannochloropsis sp.* primarily employs nitrogen assimilation mechanisms, including the uptake of ammonium (NH_4^+) and nitrate (NO_3^-), and the use of these components in enzymatic pathways for amino acid and protein synthesis [74]. Under nitrogen-limited conditions, *Nannochloropsis sp.* shows metabolic adaptation by increasing lipid production and reducing nitrogen requirements [75]. The ability of the cheese production process to grow in high-nitrogen environments such as wastewater demonstrates its effectiveness in bioremediation processes [76]. On the other hand, *Nannochloropsis sp.* is a microalgae species that can uptake inorganic phosphate even at low phosphorus concentrations due to its high-affinity phosphate transporters [77]. Additionally, it regulates phospholipid synthesis by adapting to phosphorus concentration levels and optimizes energy production under low phosphorus conditions [78]. These mechanisms demonstrate that *Nannochloropsis sp.* is a strong candidate for sustainable nitrogen and phosphorus removal and biomass production.

Fig. 5 depicts actual values found from experimental studies against predicted values generated from the model for COD, TOC, TKN, Ortho-P in microalgae-based wastewater systems. The correlation can be seen in the linear relationship between the predicted and actual values. As seen from Fig. 5, the values were distributed around the linear line except TKN. R^2 values were found to be approximately 0.97, 0.92, 0.63 and 0.92 for COD, TOC, TKN, Ortho-P, respectively. The values indicated that the predicted values by the model have a good fit with the experimental data except TKN. R^2-adjusted values were found to be approximately 0.96, 0.87, 0.36 and 0.87 for COD, TOC, TKN, Ortho-P, respectively. In biological systems, models for nutrient parameters like TKN have shown lower R^2 values compared to COD and have faced challenges in prediction accuracy [79]. However, in microalgae-based wastewater treatment studies, an R^2 value above 0.5 represents an acceptable level of accuracy [80]. Despite being a powerful experimental design and optimization method, RSM has some limitations in fully representing nonlinear and complex interactions among parameters [81,82]. Overall, it could be concluded that the model can be accepted as reliable for most of the pollutant parameters.

It has been stated that although the role of autotrophic/heterotrophic shares in nutrient removal is not entirely understood, mixotrophic microalgae have a superior potential for wastewater nutrient removal [83]. Mayers et al. [56] stated that the nutrient content of the anaerobically digested food waste can be utilized by *Nannochloropsis* sp. especially in terms of the nitrogen. However, the organic content of the waste was not evaluated in this study. *Nannochloropsis sp.,* which is a

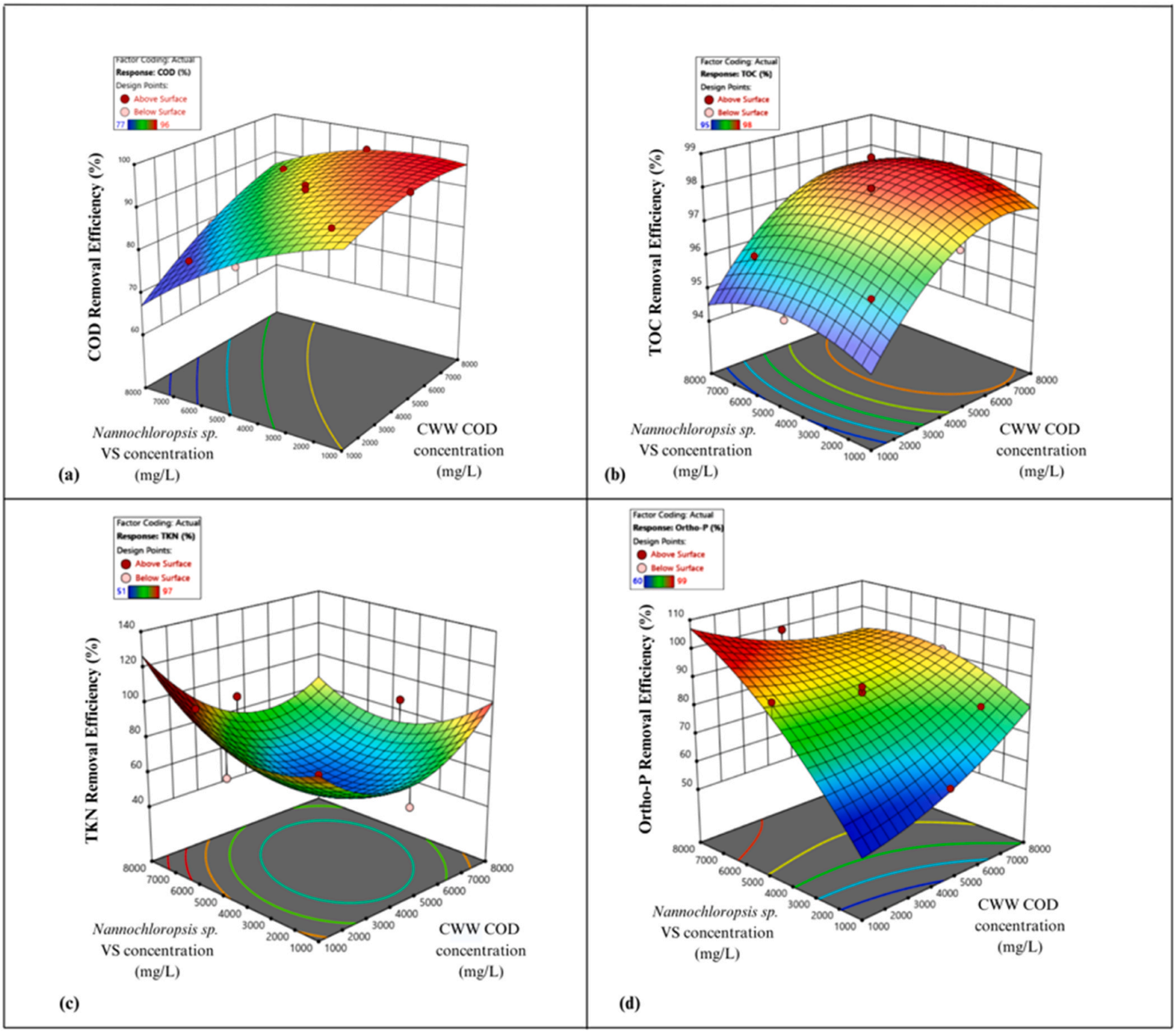

Fig. 4. 3D Surface graphs of (a) COD; (b) TOC; (c) TKN; and (d) Ortho-P removal rates in microalgae-CWW reactors.

mixotrophic microalgae, has been shown to have a potential for both carbon and nutrient removal from CWW. Caporgno et al. [84] cultivated three different microalgae including two freshwater species, *Chlorella kessleri* and *Chlorella vulgaris*, and marine microalgae species, *Nannochloropsis oculata* using urban wastewater. Freshwater species have demonstrated high efficacy in nutrient removal from wastewater, but marine species necessitate acclimatization or optimization to achieve enhanced nutrient removal or increased biomass production for important chemicals [84]. In our study, different CWW and microalgae concentrations were selected according to RSM. Notably, reactors featuring elevated concentrations of *Nannochloropsis sp.* exhibited enhanced nutrient removal (TKN and Ortho-P), whereas reactors with higher CWW concentrations demonstrated greater efficacy in carbon removal (COD and TOC). The results support that it is critical to optimize the microalgae-based systems using methods such as RSM to achieve high removal efficiencies in terms of the various parameters when treating wastewaters.

Table 3 summarizes the analysis of variance (ANOVA) results for the predictive model and the values showing the correlation. The p-values were less than 0.05, indicating a 95 % confidence interval except for the TKN parameter. R^2 (predicted) refers to how well the model predicts for new data. The fact that the R^2 (predicted) value is lower than the R^2 value supports the compatibility of the model. If the regression model cannot adequately explain the relationship between the response variable and the model, this is explained by the "lack of fit" value. If the lack of fit value is exceeds 0.05, the model and data are compatible with each other, but if it is smaller, it indicates that the model does not fit the data. The lack of fit value exceeded 0.05 for all parameters except TKN. The results indicated the suitability of the model except for TKN parameters.

The RSM was generated using this data. The forecasting models for the removal of COD (Eq. 2), TOC (Eq. 3), TKN (Eq. 4) and Ortho-P (Eq. 5) were given, respectively, as follows:

$$\text{COD \%} = 90.40 + 0.002171\, CWW - 0.001215\, MA - 0.000000\, CWW * CWW - 0.000000\, MA * MA + 0.000000\, CWW * MA \quad (2)$$

$$\text{TOC \%} = 93.466 + 0.001077\, CWW + 0.000444\, MA - 0.000000\, CWW * CWW - 0.000000\, MA * MA + 0.000000\, CWW * MA \quad (3)$$

$$\text{TKN \%} = 119.5 - 0.01819\, CWW - 0.00959\, MA + 0.000002\, CWW * CWW + 0.000002\, MA * MA - 0.000001\, CWW * MA \quad (4)$$

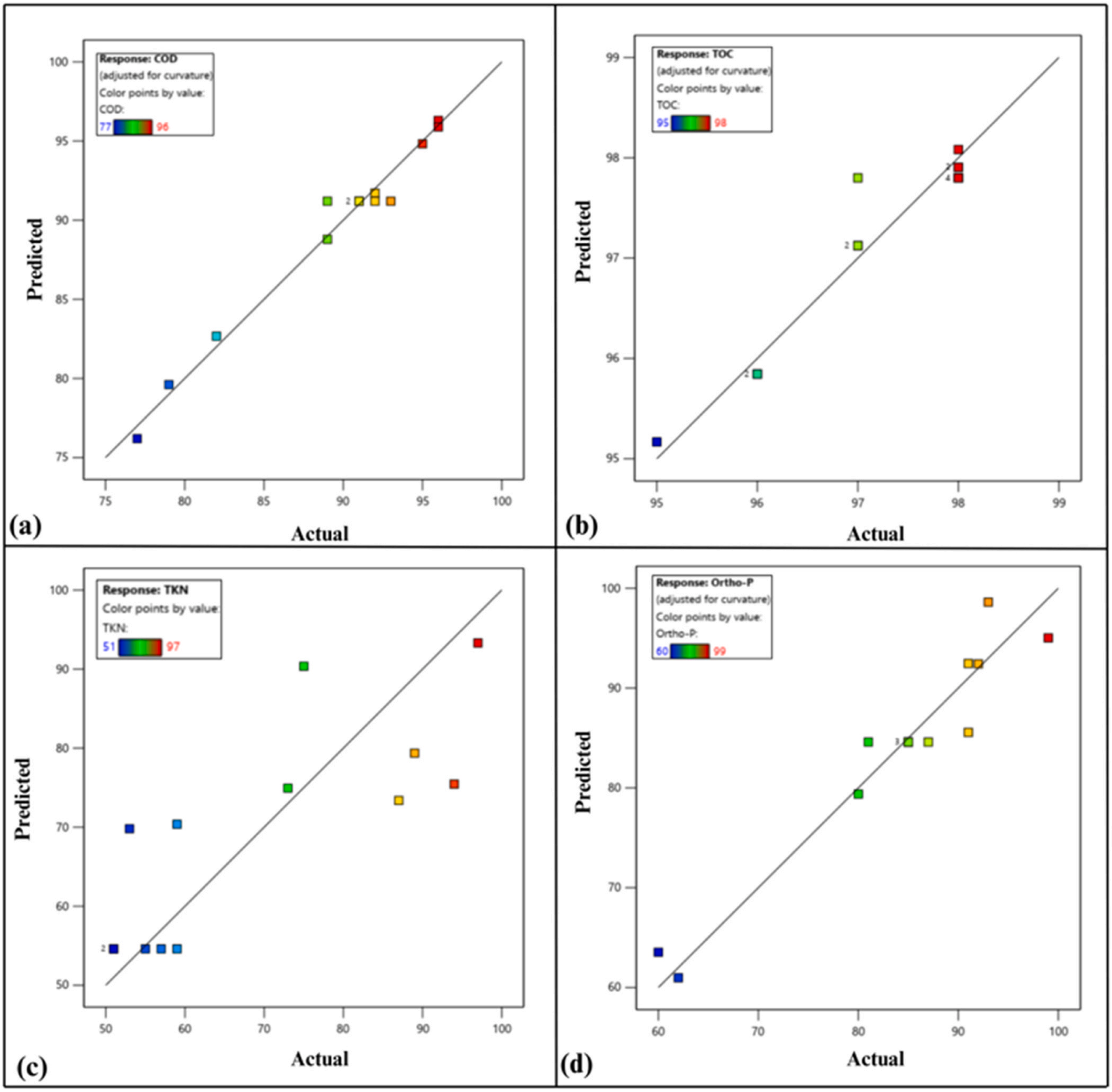

Fig. 5. Predicted and actual values of removal efficiency (a) COD; (b) TOC; (c) TKN; and (d) Ortho-P.

Table 3
ANOVA model responses for COD, TOC, TKN and Ortho-P removal efficiencies.

	COD	*TOC*	*TKN*	*Ortho-P*
Model				
Sum of Squares	456.54	11.38	2289.68	1452.67
Df	5	5	5	5
Mean Square	91.31	2.28	457.94	290.53
F-value	60.68	17.08	2.39	18.12
p-value	< 0.01	0.01	0.14	0.01
Std. Dev.	1.23	0.36	13.83	4
R^2	0.97	0.92	0.63	0.92
R^2 *(adjusted)*	0.96	0.87	0.36	0.87
R^2 *(predicted)*	0.94	0.82	−1.54	0.56
Lack of fit	0.85	0.88	0.01	0.05

$$\text{Ortho-P \%} = 35.90 + 0.00205\, CWW + 0.01376\, MA + 0.000000\, CWW * CWW - 0.000001\, MA * MA - 0.000001\, CWW * MA \quad (5)$$

*CWW: Cheese whey wastewater COD concentration, MA: *Nannochloropsis sp.* microalgae volatile solids concentration

The response optimizer was performed using the software, and the outcome of Minitab was response optimization, showing the best removal efficiencies for the parameters at optimum concentrations. The results are depicted in Fig. 6. In this study, through systematic experimentation and analysis, the optimal concentration of CWW was proposed as 8000 mgCOD/L, while the optimal concentration of microalgae was found to be approximately 2200 mgVS/L. For this set, the desirability (d) values of COD, TOC, TKN, and Ortho-P parameters are determined as 0.99, 0.94, 0.78, and 0.63, respectively. A remarkable

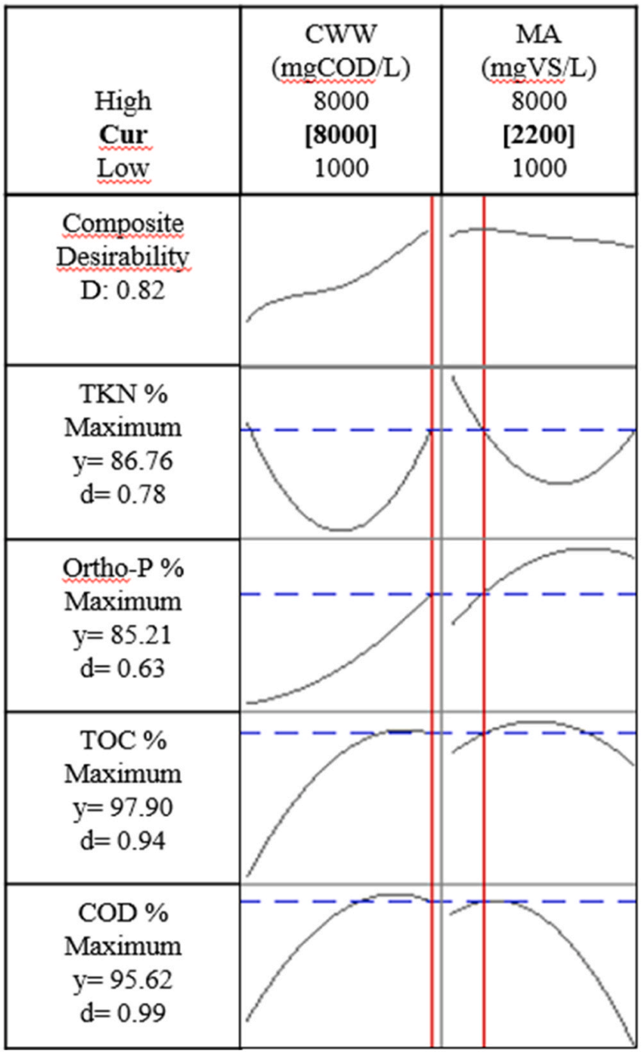

Fig. 6. Response optimizer at optimum condition for maximum goal. *CWW: Cheese whey wastewater COD concentration, MA: *Nannochloropsis sp.* microalgae volatile solids concentration.

desirability value of 0.99 for COD removal showcases the exceptional effectiveness of the applied parameter settings. This near-optimal result suggests that the chosen conditions have led to an impressive reduction in organic load within the wastewater. The achieved desirability value of 0.78 for TKN removal indicates a substantial success in the optimization process. The results suggest that the chosen combination of parameter settings has led to an effective reduction in TKN levels within the CWW. Ortho-phosphate removal, as indicated by the desirability of 0.63, demonstrates the capacity of microalgae-based treatment to attenuate phosphorus content in CWW. The COD concentration of CWW and volatile solids concentrations of *Nannochloropsis sp.* microalgae were taken into consideration for the removal efficiency of all parameters (TKN, COD, TOC, and Ortho-P). The detailed evaluation of several parameters, such as light intensity, light cycle, and temperature for the microalgae, can further increase removal of these parameters. Therefore, the overall optimization of all parameters could positively affect the desirability value in the model.

The experimental data obtained from laboratory sets prepared with different microalgae and CWW ratios for removing COD, TOC, TKN, and Ortho-P were used to develop an optimal RSM model. Table 4 presents the observed and predicted %R (Eq. 6) [85] values for COD, TOC, TKN, and Ortho-P using the RSM model, along with the corresponding relative error percentages (Eq. 7) [85]. As seen from the table, the model has the lowest relative errors for Ortho-P and TKN.

Table 4
Experimental and predicted values of %R for COD, TOC, TKN, and Ortho-P and Relative Error values (%).

Parameter	Experimental value (% R)	Predicted value (% R)	Relative Error (%)
COD	90.2 ± 1.3	105.09 *	−16.51
TOC	81.8 ± 1.8	103.05 *	−25.97
TKN	66.7 ± 8.6	72.96	−9.38
Ortho-P	64.8 ± 2.9	60.13	7.2

*COD/TOC removal rate can be maximum 100.

$$\%R = (C_0 - C_1)/C_0 * 100 \quad (6)$$

where;

C_0 represents the concentration of all parameters before treatment (mg/L).

C_1 indicates the concentration of all parameters after treatment (mg/L).

% R denotes the percentage removal of the selected parameters.

$$\text{Relative Error (\%)} = (Yexp \quad .-Yp.)/Y\,exp. * 100 \quad (7)$$

In this study, RSM, which is also widely used in the literature for optimization studies, has been employed and it was found that the critical parameter for carbon removal was only the CWW concentration, while the critical factors for nutrient removal were both the CWW concentration and the *Nannochloropsis sp.* concentration. RSM determined the important parameters such as microalgae and CWW concentration for the removal of carbon and nutrients, however the method was unable to determine the critical level of the concentration of these parameters. In contrast, a decision tree clearly highlights this situation by providing critical concentration levels. In a study by Sabour and Amiri [86], RSM was initially applied in leachate treatment, followed by the evaluation of the model's accuracy and predictive power using an ML algorithm called ANN (Artificial Neural Networks). As a result, it was shown that applying ANN after RSM improved ANN's predictive performance. Therefore, in our study, Decision Tree, was used to evaluate the accuracy of the results obtained with RSM for the predictive power of the model.

3.3. Decision tree

Since RSM has some limitations, machine learning has been used complementarily to this method in this study to test the accuracy of the model and obtain more information. Decision Tree has been used to test the reliability of the data obtained from RSM. Decision Tree can contribute to a more comprehensive evaluation of the results predicted by RSM by analyzing the relationships between variables in a hierarchical structure and can enhance the model's reliability [87].

To determine the most appropriate MA and CWW concentrations, decision tree analysis was performed by taking into account the removal efficiencies of pollutant parameters in wastewater. Fig. 7 illustrates the decision tree based on independent variables that enable *Nannochloropsis sp.* to increase biomass in CWW. The critical parameters influencing biomass growth were tested as CWW concentration and *Nannochloropsis sp.* concentration. The model obtained the highest COD removal efficiency (96 %) when the CWW concentration was approximately 7500 mg COD/L, but the *Nannochloropsis sp.* concentration was not critical for this high removal efficiency. The concentration values obtained for the highest removal efficiency (98 %) for TOC, another carbon removal parameter, yielded identical results to those for COD. In contrast to carbon removal, the removal efficiency for nutrient removal (TKN and Ortho-P) was positively influenced by low CWW concentration and high *Nannochloropsis sp.* concentration. In other words, while a single parameter (CWW concentration) proved effective in carbon

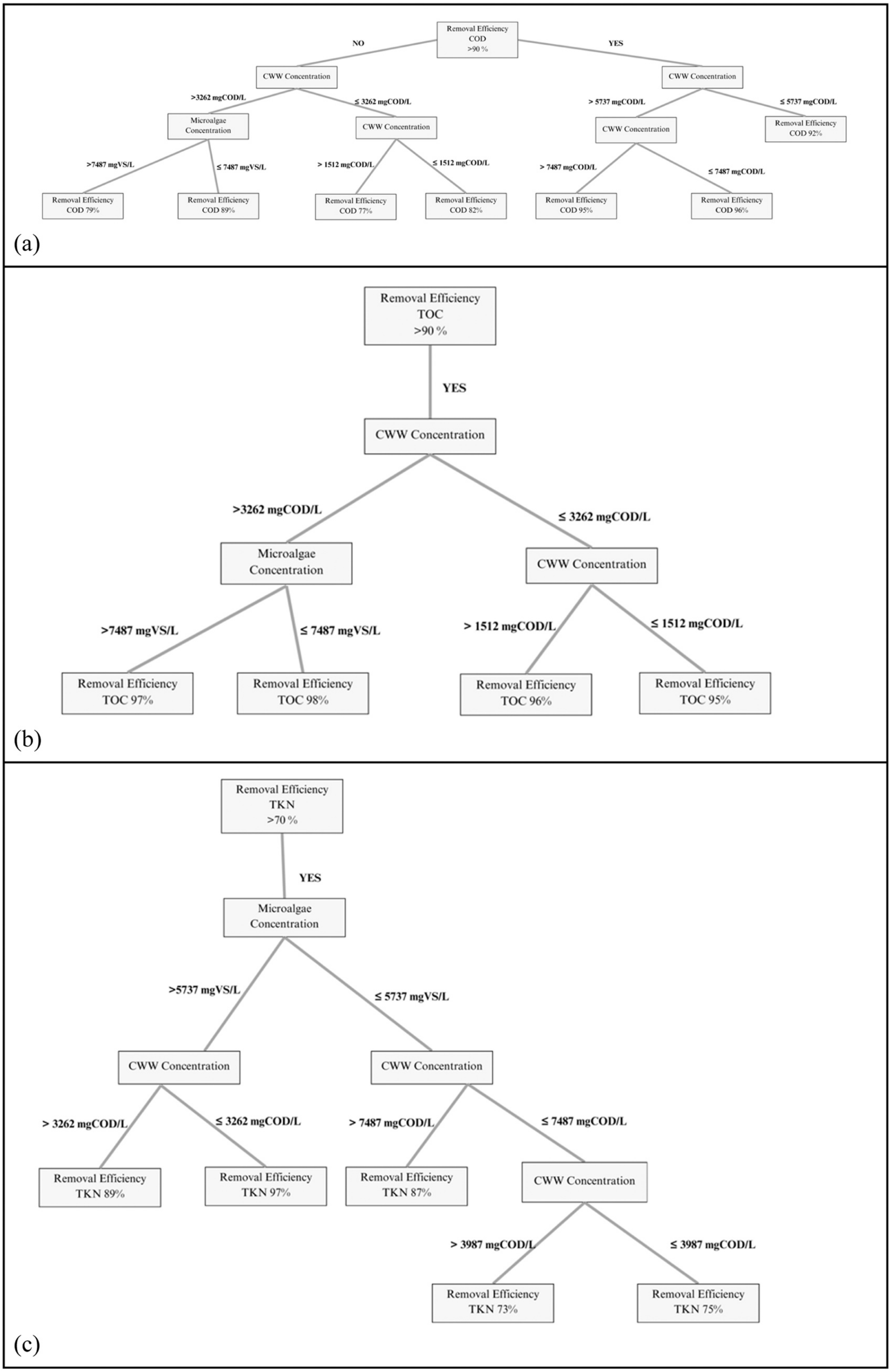

Fig. 7. Decision trees that illustrate the removal efficiencies of (a) COD; (b) TOC; (c) TKN; and (d) Ortho-P in CWW.

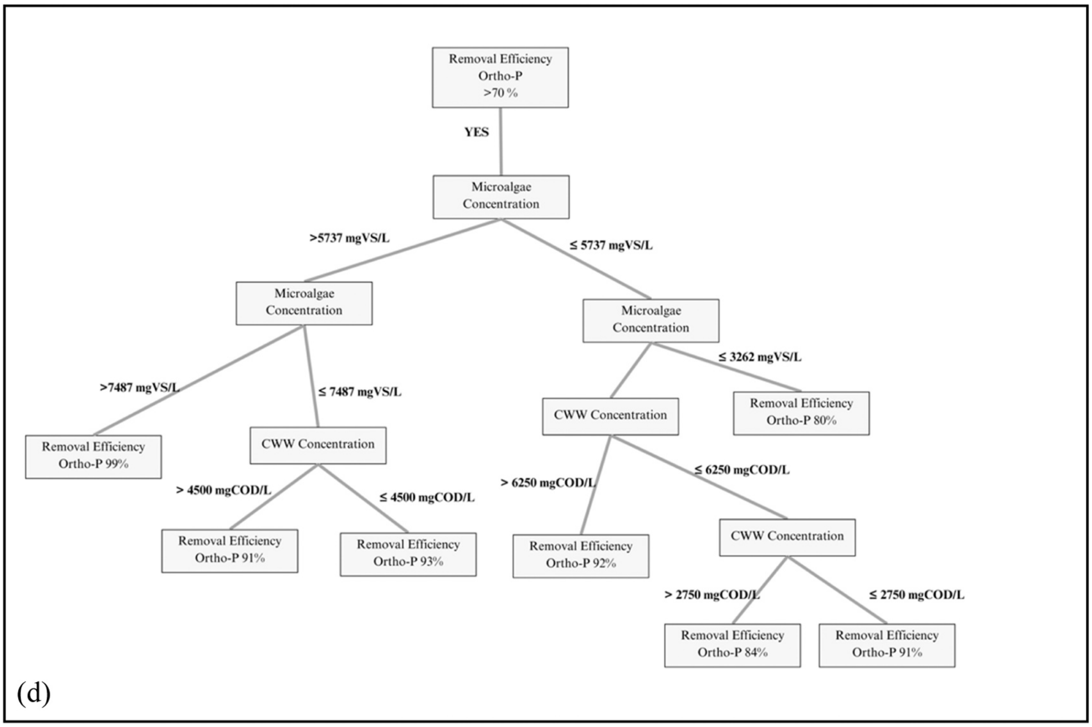

Fig. 7. (*continued*).

removal, evaluating both parameters simultaneously is necessary for nutrient removal. When *Nannochloropsis sp.* concentration exceeded approximately 5700 mgVS/L and CWW concentration fell below approximately 3250 mgCOD/L, the highest removal efficiency (97 %) has been reported for TKN. For Ortho-P, high removal efficiency (>93 %) was required when *Nannochloropsis sp.* concentration was above approximately 5700 and CWW concentration was less than 4500 mgCOD/L. The critical parameters for nutrient removal differ from those for carbon removal. Because the nutrient (nitrogen and phosphorus) removal mechanism of mixotrophic microalgae is explained by their capacity for direct utilization [88]; the carbon removal mechanism is associated with photosynthesis and the presence of organic carbon in the environment [89]. Although both mechanisms support microalgal biomass production, they differ in terms of biochemical processes and environmental requirements [23,90]. These microalgae species can activate the carbon removal mechanism even at low microalgal biomass concentrations [91].The RSM analyses failed to clearly understand the level of both variables for nutrient removal. However, the decision tree clarified this distinction. We observed that in this system with a higher carbon removal rate, the CWW concentration, which influences carbon removal, ranked highly in the evaluation of optimal concentrations.

A number of studies show that various properties significantly affect microalgae performance in wastewater treatment. It is obvious that the exact determination of optimum values for these parameters by various methods improves the overall accuracy of the process. Since it is a very difficult and long process to analyze all the parameters affecting the process one by one using experimental setups, the most important parameters such as the light intensity (5000 lux) and temperature (24 °C) values [92], which were previously reported in the literature with *Nannochloropsis sp.,* were applied in this study. However, by focusing on the concentration values, our study successfully predicted *Nannochloropsis sp.* microalgae in CWW, and it differed from Singh *et al.* [93] study in terms of wastewater content, parameters chosen, and microalgae species used. This decision tree algorithm facilitates the treatment of wastewaters that are normally very difficult to treat and provides solutions. It can shorten the time required to identify important process parameters and can be a precursor for the treatment of other wastewaters that are normally very difficult to treat. Gowd *et al.* [94] study, which used dairy sector wastewater and microalgae (*Scenedesmus sp.* and *Chlorella sp.*), recorded the highest influent wastewater COD value at 3488 mg/L, which is significantly lower than the 8000 mg/L observed in our study. Meenatchisundaram *et al.* [95] compared seven different machine learning models for microalgae-based wastewater and found that the maximum COD input was 350 mg/L, which is quite low compared to our study.

3.4. Chlorophyll-a change

In this study, chlorophyll-a which is found in all microalgae species has been monitored by the spectrophotometer at 680 nm during the study. It has been found that chlorophyll-a showed an increase in all reactors (Fig. 8). However, the increase varies for each reactor depending on the ratio of the CWW/MA. The highest chlorophyll-a increase occurred in the reactor with CWW/MA ratio of 4.5 (CWW:4500 mgCOD/L; MA:1000 mgVS/L). The results are parallel with the ratio at which the optimum values are determined. At the optimum concentration, i.e. CWW: 8000 mgCOD/L and MA: 2202 mgVS/L, the CWW/MA ratio was found to be 3.63. Generally, it can be seen that chlorophyll values are found to be higher at high CWW/MA ratios compared to the other ratios. Several studies highlight the presence of a positive correlation between N-P availability and chlorophyll content in mixotrophic microalgae [96]. It has been also claimed that the growth of marine microalgae such as *Chlorella* sp. and *Nannochloropsis sp.* was positively affected by the exposure to the light and elevated glucose concentration [97].

It has been stated that the microalgae concentration has a major role in ammonia removal and the rate of the removal is positively affected by the optimum microalgae concentration [35]. In our study, a similar trend was also seen for TKN removal. For the same wastewater concentrations (4500 mg COD/L), TKN removal was 51 % at a microalgae concentration of 4500 mgVS/L, whereas it increased to 89 % when the microalgae concentration was raised to 8000 mgVS/L. This result shows a positive correlation between *Nannochloropsis sp.* concentration and TKN removal. However, in another study, it has been reported that

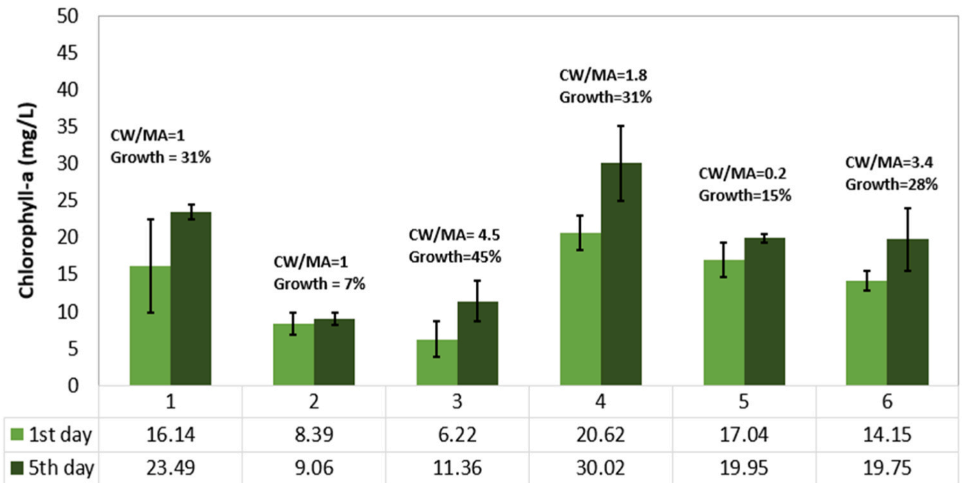

Fig. 8. Chlorophyll-a levels in the reactors. * (Reactor (R)1: 4500 mgCOD/L CWW- 4500 mgVS/L *Nannochloropsis sp.* (*NP*); R2 2025 mgCOD/L CWW-2025 mgVS/L *NP*.; R3 4500 mgCOD/L CWW 1000 mgVS/L *NP*; R4 8000 mgCOD/L CWW- 4500 mgVS/L *NP*; R5: 1000 mgCOD/L CWW-4500 mgVS/L *NP*; R6:6975 mgCOD/L CWW-2025 mgVS/L *NP*.).

extremely low and extremely high concentrations of microalgae resulted in rate limiting and significantly impacted ammonia removal [35]. Therefore, it can be concluded that microalgae concentration is one of the determining factors for effective TKN removal from the wastewater and the initial ratio of the microalgae concentration should be carefully selected. For the same microalgae (*Nannochloropsis sp.*) concentration (6975 mgVS/L), the TKN removal efficiency was 97 % with a CWW concentration of 2025 mgCOD/L, compared to 53 % TKN removal at a CWW concentration of 6975 mgCOD/L. This showed that even if the microalgae concentration is high, the concentration of CWW fed to the reactor is also an important parameter in the removal efficiency.

4. Conclusions

In this study, initially, the growth of *Nannochloropsis sp.* microalgae fed with CWW along with the removal efficiencies of different pollutants was investigated using the RSM optimization. It has been shown that marine microalgae strains like *Nannochloropsis sp.* could be produced using saline CWW in bioreactors. In reactors with high concentrations of *Nannochloropsis sp.,* nutrients (TKN and Ortho-P) were removed more efficiently, while reactors fed with higher CWW concentrations exhibited better efficiency in carbon removal (COD and TOC). This study demonstrates the importance of optimizing microalgae and influent wastewater concentrations using methods such as RSM to achieve high treatment efficiency from microalgae-based systems. However, RSM analyses are limited in clearly showing the relative importance of variables when evaluated together on nutrient removal. In contrast, the decision tree model provided a clear understanding of these distinctions by effectively distinguishing the key factors influencing nutrient removal. The findings highlight the potential of the decision tree as a complementary tool to RSM, particularly in visualizing variable interactions and making interpretable decisions in wastewater treatment optimization. For future studies, further research and development is essential to ensure the successful integration of this technology into practical wastewater treatment systems. To scale up the systems, RSM and decision tree can be integrated into microalgae-based wastewater treatment systems in order to determine optimum operating conditions and the ideal ratios of microalgae/influent wastewater concentrations, thereby enhancing the target pollutant removal. Future research should focus on long-term performance evaluations to assess the resilience and stability of microalgae-based systems under various operating conditions in real-world scenarios. When transitioning to large-scale implementation, it is critically important to consider economic sustainability, regulatory compliance, and environmental impact. Additionally, exploring a wider range of applications for the biomass produced by the treatment processes, such as the production of biofuels and bioplastics etc., offers exciting prospects for a sustainable future.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Not applicable

Consent for publication

The authors consent to publish the article on acceptance.

Funding

No funding was received to assist with the preparation of this manuscript.

CRediT authorship contribution statement

Kayan Iremsu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **AYMAN OZ Nilgun:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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