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Optimization of the Biodegradation of Perfluorooctanoic Acid (PFOA) and Perfluorooctane Sulfonate (PFOS) using the Activated Sludge Microbiomes Through the Box-Behnken Design (BBD)

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Abstract

The bacteria community from the aerobic zone in the activated sludge at Zeekoegat wastewater treatment plant (WWTP) was used to assess the removal of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in aqueous solution under specific conditions. The bioremediation experiment of these compounds was investigated by a batch experiment. All parameters (such as the initial pH, initial inoculum, and initial concentration), that could affect the bioremediation experiment of PFOA and PFOS were studied. The Box-Behnken Design (BBD) and the effects of these three parameters were evaluated. The results and several coefficients showed that the obtained model was acceptable for predicting the PFOA and PFOS removal efficiency using the activated sludge microbiome. Optimum conditions were determined by means of variance analysis (ANOVA), using the BBD under response surface methodology developed by Design Expert 13.0.1.0 software program. Based on the results obtained from the application of response surface methodology, a quadratic and a linear model were developed for PFOA and PFOS, respectively. From the ANOVA, the most influential factors on each experimental design response were identified. After optimizing various parameters, the predicted removal efficiency was found to closely agree with the experimental values. Optimal conditions for PFOS removal using activated sludge microbiome were determined to be pH 4, initial concen-

tration of 50005 ng/L, and initial inoculum concentration of 0.05. Similarly, for PFOA removal, optimal conditions were pH 4.58, initial concentration of 97497 ng/L, and initial inoculum concentration of 0.103. At these optimized conditions, PFOS and PFOA exhibited removal efficiencies of 72% and 93%, respectively, with desirability values of 0.96 and 0.99. Allowing a possible practical application in future water treatment

Keywords: Box-Behnken Design; PFOA; PFOS; Percentage Removal; Optimization

Introduction

Long chain per- and poly-fluoroalkyl substances (PFASs) such as the eight-carbon homologues perfluorooctanoic acid (PFOA) and pefluoroalkyl sulfonate (PFOS), are receiving attention due to their persistence, bioaccumulation potential and adverse effects on biota and humans [1]. They are man--made and high-volume industrial chemicals, which have been detected in water, soil, atmosphere and wildlife in many countries across the globe, including in South Africa [2]. During the past 50 years, PFOA and PFOS have been used as lubricants, surfactants, fire retardants, and polymer additives in many industries and household applications [3]. However, they tend to break down very slowly in the natural environment, and as a result, they have been added to the Stockholm Convention lists of persistent organic pollutants [4]. Adverse health effects occur, when expose to these chemicals at a concentration of 0.07 ppb (70 ppt) [5]. Another study by [6] reported a concentration of up to 19.2 µg/L for PFOA in surface water near an industrial zone. Furthermore, a higher concentration of 1000 mg/L (PFOA) was detected in wastewater from diluted sample as reported by [7]. Based on the above, there is a concern in developing new cheap technologies with high sorption capacity to remove PFOA and PFOS from polluted water. Many process technologies for the treatment of PFOA and PFOS in the environment have been reported. However, it has been reported by previous authors that most conventional degradation processes are ineffective for the degradation of these compounds, because of the high energy carbon– fluorine which are present in their molecule making it inherently recalcitrant to chemical and biodegradation treatments [8-12]. Several studies have reported the bioremediation of these emerging contaminants using pure bacterial culture under various environmental conditions [11,13-15]. However, these studies were sensitive to environmental changes and no complete mineralisation was observed. It will be reasonable to gain insight using a bacterial consortium that might utilise and ultimately mineralise these emerging pollutants.

Environmental and operational factors can affect microorganisms and/or impact microbial community function.

Generally, bioremediation is affected by several factors such as nutrient levels, pH, temperature, air composition, nutrient availability, bacterial composition, types of growth media and the concentrations of pollutants [16]. These parameters are also important when determining the degradation rate of PFAS compounds [17]. For example, understanding pH homeostasis may have implications and applications in fields as diverse as bioremediation assays or the behaviour of pathogenic bacteria [18]. The initial concentration of PFOA and PFOS in wastewater can influence their biotransformation. Higher concentrations of these compounds can greatly affect microbial degradation, and at extremely high concentrations, the microbial community may be overwhelmed, leading to slower biotransformation rates or inhibition of biodegradation process by the microbes used [19]. Another study pointed out that when the bioremediating species were subject to specific conditions such as enhancement with exogenous carbon sources, and under resting states in the case of aerobic biodegradation and for anaerobic bacteria, the supply for suitable electron donors for anaerobic metabolisms, the efficiency of PFAS' removal could be increased [20]. Furthermore, it is well acknowledged that pH is a major driver controlling microbial communities in terrestrial ecosystems [21,22], It is therefore important to identify optimal conditions that support microbial activity and growth that are essential for efficient biotrans-

formation. The current study uses the Box-Behnken design (BBD) in the optimization of experiments using RSM [23] to assess the effect of important parameters and their interactions on the removal efficiency of PFOA and PFOS using activated sludge microbiome. Parameters such as initial concentration, initial pH, and initial inoculum concentration can affect the removal efficiency of PFOA and PFOS. Batch experiments were conducted using the consortium of bacteria from the aerobic zone activated sludge compartment at Zeekoegat WWTP. To date, study reporting PFOA and PFOS removal with a bacteria consortium from the aerobic zone compartment in the activated sludge is limited. In this study, the effect of initial dosage concentration, initial pH, and initial inoculum concentration were tested. Moreover, the response surface methodology was adopted to discover the key parameters affecting PFOA and PFOS removal efficiency.

Materials and Methods

Materials

The standards of PFOA and PFOS (50 µg/mL) prepared in methanol (MeOH) were purchased from Wellington Laboratories (Ontario, Canada). Ammonium acetate ($C_2H_7NO_2$) (used as mobile phase additive), methanol (MeOH) (LC-MS grade) and water (LC-MS grade) were purchased from Sigma Aldrich (Aston Manor, South Africa). Internal standard of octanoic acid (M_2PFOA) was also purchased from Wellington Laboratories (Ontario, Canada). Solid phase extraction (SPE) Oasis® hydrophilic-lipophilic balance (HLB) (500 mg, 12 mL) cartridges and membrane filter (pore size 3, 1 and 0.22 µm) were also purchased from Sigma-Aldrich (Aston Manor, South Africa).

Primary stock solutions of the targeted PFASs were prepared in methanol at a concentration of 1 mg/L. Working standard mixture solutions were prepared using appropriate dilution of the stock solutions in MeOH. Furthermore, the prepared working standard solutions were used for the preparation of the calibration curves and for spiking samples in the validation study. All solutions were stored at 4 °C in amber glassware to prevent light degradation.

The aerobic sludge from Zeekoegat WWTP in Pretoria South Africa, was used as the inoculum source in the present study. The experiment was performed using a minimal medium (M63) prepared using the following $(NH_4)_2SO_4$, 2.0 g; KH_2PO_4, 13.6 g; $FeSO_4.7H_2O$, 0.5 g; after autoclaving the followings additional supplements were added: 10 mL of a sterile 20 % solution of glycerol and 1 mL of a sterile 1M $MgSO_4$ solution, expressed in g/L according to [24].

Design of Response Surface Experiment (Box Behnken Design (BBD)

The Box-Behnken design (BBD) as the most widely used type of Surface Response Method (RSM) was applied with three independent parameters of initial pH (A), Inoculum concentration (B), Initial concentration (C) for modelling, optimizing, and determining the effect of these independent parameters and the simultaneous interactions of these parameters on the response function (removal efficiency of PFOA and PFOS by activated sludge microbiome) using Deign-Expert 13.0.0 software. In this study three parameters were studied, such as the initial pH (4-9) (A), Inoculum concentration (0.05-0.28) (B), Initial concentration (10-100000 ng/L) (C) for PFOA; and the initial pH (4-9) (A), Inoculum concentration (0.05-0.32) (B), Initial concentration (10-100000 ng/L) (C) for PFOS. The model was made of three levels (low, medium, and high, being coded as -1, 0, and 1) and the response in this study was the percentage removal of the pollutants. These three variables along with their respective ranges were chosen based on the literature and our preliminary experimental study.

In response surface methodology (RSM), a second-degree equation is a specific type of polynomial model used to represent the relationship between the response variable and the input variables. This equation allows for the examination of quadratic effects and interactions between the factors. The general form of a second-degree equation in RSM is given by Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 + \varepsilon \quad (1)$$

Where:

Y is the response variable,

X_1, X_2 are the input variables (factors),

β_0, β_1, β_2, β_3, β_4, β_5 are the regression coefficients (intercept, main effects, quadratic effects, and interaction effect),

ε represents the residual or error term.

The linear terms (β_1X_1, β_2X_2) in this equation capture the main effects of the factors, while the quadratic terms ($\beta_3X_1^2$, $\beta_4X_2^2$) represent the curvature of the response surface. However, the interaction term ($\beta_5X_1X_2$) accounts for the combined effect of the two factors. To estimate the coefficients β_0, β_1, β_2, β_3, β_4, β_5, data is collected by conducting experiments at different levels of the factors [25]. By analysing the second-degree equation, researchers can gain insights into the curvature of the response surface as well as the interactions between factors. This information can be applied to optimize the system being studied, identify optimal factor settings, and understand the relationship between the factors and the response variable in more detail [26].

Table 1: Level of various independent variables at coded values of response surface methodology experimental design for PFOA

Symbol	Independent Variables	Coded Levels		
		-1	0	1
A	Initial pH	4	6	9
B	Initial Inoculum	0.05	0.165	0.28
C	Initial Concentration (ng/l)	10	50005	100000

The initial pH, the inoculum concentration, and the initial pollutant concentration were found to be significant variables affecting the removal efficiency of PFOA and PFOS using the activated sludge microbiome. These variables together with their values are summarised in Table 1 and Table 2 for the PFOA and PFOS, respectively. A total of 17 runs were generated from the model.

Table 2: Level of various independent variables at coded values of response surface methodology experimental design for PFOS

Symbol	Independent Variables	Coded Levels		
		-1	0	1
A	Initial pH	4	6	9
B	Initial Inoculum	0.05	0.185	0.32
C	Initial Concentration (ng/L)	10	50005	100000

The optimization of the growth factors was determined using the BBD model and parameters such as pH, initial inoculum concentration and initial pollutant concentrations were monitored. This was achieved by incubating the isolates using the generated parameters from the Box-Behnken in the MM media. Seventeen (17) samples in duplicate were prepared using specific parameters, subsequently a series of experiments were conducted in 10 mL test tubes and incubated at room temperature in a shaking incubator for 48 hours, to assess the removal efficiency of PFOA and PFOS using the bacteria consortium. Samples were extracted using solid phase extraction (SPE) prior to LC/MS/MS analysis. The pH of the media was adjusted using a pH meter by adding hydrochloric acid or sodium hydroxide solutions. After the quantification of PFOA and PFOS concentration by LC/MS/MS, the removal efficiency was determined using Eq. (2) bellow.

$$Removal\ efficiency\ (\%) = C\ influent - C\ effluent\ /100 \quad (2)$$

Where:

C influent and C effluent represent the concentration in ng/L of the chemicals before and after incubation, respectively.

Sample Pretreatment and Solid Phase Extraction (SPE)

A solid phase extraction technique utilizing a vacuum manifold, which can hold up to twelve SPE Oasis® HLB (500 mg, 12 mL) cartridges, was used for sample pretreatment before the quantification analysis with the liquid chromatography tandem-mass spectrometry (LC/MS/MS). All the sample were prepared in triplicates. The extraction technique was based on the modified method of [27]. In summary, the SPE cartridges were conditioned using 4 mL of MeOH followed by 4 mL of Milli-Q water. Afterward, 10 mL of the samples were loaded onto the pre-conditioned cartridges and extracted. The sample holders were dried under vacuum suction for 20 min. Once extraction was completed, analytes were eluted with 6 mL of MeOH at a very low flow rate of 1 drop/s. After extraction, the eluate was concentrated to near dryness under a gentle nitrogen stream and reconstituted to a volume of 1 mL with MeOH and 100 µL of 2000 ng/mL internal standard (M_2PFOA). The final extract was centrifuged and quantitatively transferred to 1 mL brown vials prior to LC-MS analysis.

Instrumental Analysis using Liquid Chromatography Tandem-mass Spectrometry (LC/MS/MS)

The quantitative analysis was performed using a liquid chromatography tandem mass spectrometry technique employing a Shimadzu LC-MS-8030 triple quadrupole system, Tokyo, Japan. The instrument was equipped with an electrospray ionization (ESI) source and operated in negative mode. The multiple reaction monitoring (MRM) transition for the targeted PFASs was optimized using flow injection analysis (FIA) and their fragment ions (M-H) were monitored. High concentration of the standards of 1,000 ng/L of each of the targeted PFASs were used for the optimization of MRM conditions. The mobile phase consisted of a mixture of 20 Mm ammonium acetate and 100% methanol, in HPLC grade water. Separation of the PFOA and PFOS was done on a C18 polar column. MRM transition for PFOA and PFOS and the precursor and product ions and their collision energies as well as the cone voltages are presented in Table 3. Chromatograms of the studied compounds are also presented in Annexure.

Table 3: Optimum MRM parameters for the PFAS analysis

Compounds	Precursor (m/z)	Product (m/z)	CE
PFOA	413.00	368.95	10.0
PFOS	499.00	80.15	47.0

Quality Assurance and Quality Control (QA/QC)

A series of analytical method was performed to assess the linearity and spiking experiments to validate the accuracy of the method. In this study, spiking experiment were undertaken following the same procedure as mentioned in 2.3. The extraction efficiencies were evaluated when blanks samples (ultrapure water) were spiked with a known concentration (100 ng/mL) of the surrogate standard ($MPFHxS18O_2$). The percentage recoveries of the spiked samples ranged from 74 to 90%. The percentage relative standard deviation (RSD) was calculated for each targeted PFASs to determine the precision of the instrument, which passed the QC criteria and were than 10% (Table 4). During the preparation of real samples, procedural blanks were extracted following the same procedure used for environmental samples to check for possible sources of contamination. A 10- point calibration was created by mean of the linear regression and the calibration curves showed the correlation coefficient (R^2) of greater than 0.99 for all the targeted PFASs. The limit of detection (LOD) and the limit of quantification (LOQ) from the calibration plots were calculated using 3 and 10 times the signal-to-noise ratio, respectively (Table 4). The percentage recovery of PFOA and PFOS as well as other method validation parameters is summarised and presented in Tables 4.

Table 4: Method performance and validation parameters

Compounds	LOD (ng/L)	LOQ (ng/L)	%RSD (100 ng/L)	Mean recoveries (%)
PFOA	0.37	1.03	5.13	76
PFOS	0.025	0.089	1.45	89

Statistical Analysis

The statistical analysis was carried out using Design-Expert™ software (Version 13) (Stat Ease Inc., Minneapolis, USA). To assess the experimental data, multiple regressions were employed to evaluate the experimental data and the significance of regression coefficients was assessed using the F-test. Initially, a quadratic model incorporating linear, squared, and interaction terms was applied for data modelling. Various descriptive statistical analyses such as p-values, F-values, degrees of freedom (DF), sum of squares (SS), mean sum of squares (MSS), coefficient variation (CV), determination coefficient (R^2), adjusted determination coefficient (R^2), and correlation coefficient (R), were performed. Additionally, the pareto analysis of variance (ANOVA) was used and the significant parameters in the model were calculated to generate the ANOVA tables to assess the adequacy of the predicted model and for optimization purposes. Regression models and coefficients were employed for statistical computations and to create response surface plots.

Results

Analysis of the Box-Behnken Design (BBD) Determination of the Regression model and the Effects of Model Components

The experimental and predicted results from 17 runs conducted to investigate the effects of three independent variables (initial pH, initial inoculum, and initial concentration) on the PFOA and PFOS removal are presented in Table 5 and Table 6, respectively. Through the model fitting technique in Design Expert software version 13.01.1 it was reported that the predicted values demonstrated strong agreement with the experimental outcomes.

Table 5: Box–Behnken design and corresponding experimental and predicted responses for the PFOSA

Runs	Initial pH	Initial Inoculum	Initial Concentration (ng/L)	Response Value (PFOA, %)	
				Experimental	Predicted
1	6	0.05	100000	87.00	84.79
2	9	0.165	10	80.00	80.68
3	4	0.28	50005	75.00	71.96
4	4	0.05	50005	84.00	87.54
5	6	0.165	50005	75.60	75.60
6	6	0.28	10	85.00	87.21
7	6	0.05	10	87.00	83.72
8	9	0.165	100000	60.00	59.82
9	6	0.165	50005	73.00	75.60
10	9	0.05	50005	49.00	50.95
11	4	0.165	10	81.00	81.40
12	6	0.165	50005	68.00	75.60

13	4	0.165	100000	93.00	92.10
14	9	0.28	50005	78.00	75.55
15	6	0.165	50005	79.00	75.60
16	6	0.165	50005	83.00	75.60
17	6	0.28	100000	79.00	82.28

The ANOVA data and different coefficients for the PFOA and PFOS are presented in Table 7 and Table 8, respectively. The correlation coefficients of R-squared were observed to be 0.7926 and 0.8922 for PFOS and PFOA, respectively; corresponding to explained variations of 79.26% (PFOS) and 89.22% (PFOA). Several authors reported that *R*-squared should be at least 0.80 for a good model fit [28,29]. Additionally, the significant low P-values (< 0.0500) suggested that the developed model's F distribution is statistically significant [53]. The obtained F values of 6.44 and 6.37 for PFOA and PFOS, respectively; further validate the model's significance. Furthermore, non-significant lack-of-fit P value of (0.6212 for PFOA and 0.8361 for PFOS) implies a high predictability capacity, indicating that the model effectively explains the relationship between factors and responses (Table 7 and Table 8).- Top of Form The lack of fit value being insignificant serve as an indicator that the predictability capacity of the model is high. These values suggest that the regression model effectively explains the relationship between the three factors and the response, as demonstrated in previous studies [29].

Table 6: Box–Behnken design and corresponding experimental and predicted responses for the PFOS

Runs	Initial pH	Initial Inoculum	Initial Concentration	Response Value (PFOS, %)	
				Experimental	Predicted
1	9	0.185	100000	43.00	41.34
2	6	0.185	50005	69.00	57.16
3	4	0.32	50005	58.00	60.12
4	4	0.05	50005	70.00	71.94
5	6	0.185	50005	51.00	57.16
6	9	0.05	50005	25.00	28.30
7	6	0.05	10	68.00	61.46
8	6	0.185	50005	63.00	57.16
9	6	0.32	100000	61.00	59.36
10	6	0.32	10	67.00	60.31
11	6	0.185	50005	49.00	57.16
12	4	0.185	100000	61.00	61.50
13	6	0.185	50005	56.00	57.16
14	4	0.185	10	64.00	70.56
15	9	0.32	50005	56.00	59.41
16	6	0.05	100000	49.00	47.51
17	9	0.185	10	44.00	46.37

High F value, low p value, and high sum of squares value obtained from the model indicate that the model is significant

(Table 7 and Table 8). According to the results of ANOVA, F values were obtained as 6.44 and 6.37 for PFOA and PFOS, respectively; and p values were obtained as lower than the value of 0.05 for both PFOA and PFOS. The Prob>F value being smaller than the value 0.05 indicates that the model is significant [30]. The values of sum of squares were found as high as 1618.55 and 1738.71 for PFOA and PFOS, respectively.

Table 7 indicates that parameters A and AB are statistically significant, while B, C, AC, BC, A^2, B^2 and C^2 have value larger than 0.10, suggesting that these parameters were not significant.

Table 7: ANOVA test for the Quadratic model PFOA

Source	Sum of squares	Degree of freedom	Mean square	F values	P value Prob>F	
Model	1618.55	9	179.84	6.44	0.0113	Significant
A-Initial pH	544.5	1	544.5	19.49	0.0031	Significant
B-initial inoculum	39.89	1	39.89	1.43	0.271	
C-Initial concentration	50.59	1	50.59	1.81	0.2204	
AB	412.01	1	412.01	14.75	0.0064	Significant
AC	254.13	1	254.13	9.1	0.0195	
BC	9	1	9	0.3221	0.5881	
A2	47.67	1	47.67	1.71	0.2327	
B2	3.8	1	3.8	0.136	0.7232	
C2	266.12	1	266.12	9.53	0.0177	
Residual	195.56	7	27.94			
Lack of Fit	64.36	3	21.45	0.6541	0.6212	Not significant
Pure Error	131.2	4	32.8			
Core Total	1814.12	16				
R-squared	0.8922	Mean	77.41			
Adj R-squared	0.7536	PRESS				
Pred R-squared	0.3248	C.V. %	6.83			
Adequate precision	10.1525	Std. Dev.	5.29			

The significant parameter in Table 8, were A and AB; while B, C, AC and BC were not significant. According to [31] these insignificant parameters could be removed from the developed model.

Table 8: ANOVA test for the 2FI model PFOS

Source	Sum of squares	Degree of freedom	Mean square	F values	P value Prob>F	
Model	1738.71	6	289.78	6.37	0.0055	Significant
A-Initial pH	1474.35	1	1474.35	32.4	0.0002	Significant
B-initial inoculum	182.55	1	182.55	4.01	0.073	
C-Initial concentration	139.6	1	139.6	3.7	0.1104	
AB	470.21	1	470.21	10.33	0.0093	Significant

AC	4.12	1	4.12	0.0905	0.7697	
BC	42.25	1	42.25	0.9285	0.358	
Residual	455.06	10	45.51			
Lack of Fit	175.86	6	29.31	0.4199	0.8361	Not significant
Pure Error	279.2	4	69.8			
Core Total	2193.76	16				
R-squared	0.7926	Mean	56.12			
Adj *R*-squared	0.6681	PRESS				
Pred *R*-squared	0.4431	C.V. %	9.02			
Adequate precision	10.0834	Std. Dev.	6.75			

Fitting of Second Order Polynomial Equations and Statistical Analysis

The equation resulting from the model developed in Design Expert 13.0.1.0 software program represent the relationship among the process variables and the response of the model. Specifically, Equation (3) and Equation (4) express the predicted models for PFOA and PFOS, respectively for their removal efficiency in terms of coded factors. According to these equations, the variable A, C and AB demonstrate a positive relationship in the PFOA removal by the activated sludge microbiome, while the variable AB, AC and BC exhibited a positive relationship in the PFOS removal by the activated sludge microbiome.

$$\text{Removal efficiency (PFOA)} = 95.62+1.46(A)-218.25(B)+0.000085(C)+34.95(AB)-0.000063(AC)-0.000261-0.57(A^2)+71.83(B2)+3.18(C2) \quad (3)$$

$$\text{Removal efficiency (PFOS)} = 126.00-10.72(A)1195.10(B)-0.000212(C)+31.81(AB)+8.04002E-06(AC)+0.00048(BC) \quad (4)$$

Where A, B, and C were the coded terms for the three independent variables that have been donated, i.e., initial pH, initial inoculum, and initial concentration, respectively. According to [31], a negative sign in front of the terms indicates an opposing effect, whereas a positive sign indicated a synergistic effect. Additionally adequate precision measures the signal to noise ratio and the value higher than 4 is desirable. In this work, the ratio of 10.083 and 10.153 for PFOS and PFOS, respectively; demonstrate adequate signal and indicates a high degree of experimental reliability [32,33]. Furthermore, a coefficient of variation (CV) of less than 10 is preferred for model reproducibility. The reported CV values of 9.02 and 6.83 for PFOS and PFOA, respectively, indicate a good model fit, given that lower CV values indicate smaller the residuals relative to the predicted value as reported by [32].

Adequacy of the Models

It is crucial to verify the fitted model to ensure an accurate approximation to the actual values as highlighted by [34]. Proceeding without an analysis and optimization of the fitted response surface may lead to inaccurate results. Table 5 and Table 6 present the predicted and actual values obtained for the PFOA and PFOS removal by the activated sludge microbiome, while Figure 1a and Figure 1b provide a graphical representation of the data. The proximity of data points with the straight line in these plots indicates a significant agreement between the predicted and the experimental values, indicating the adequacy of the second-order regression model.

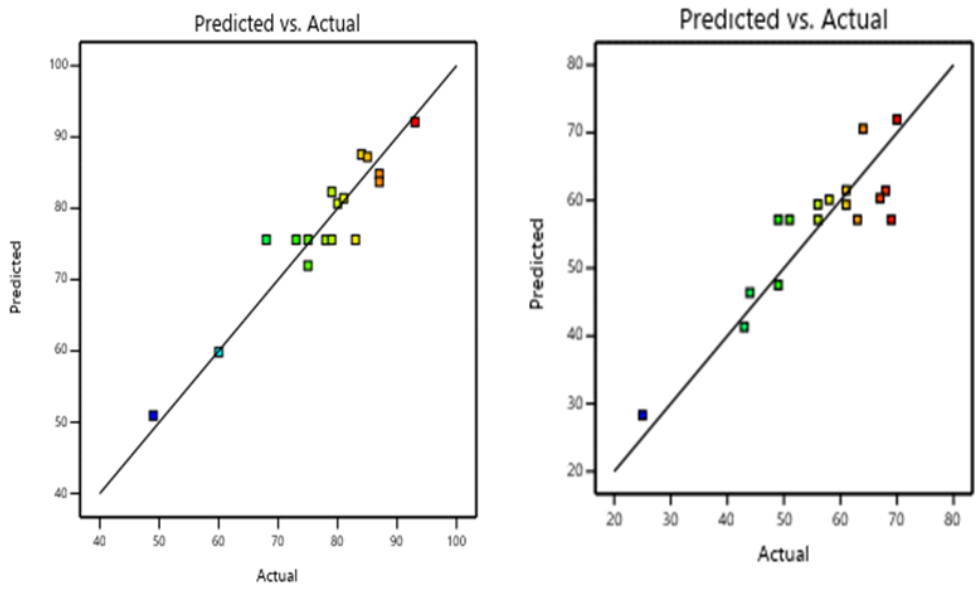

Figure 1a: Actual vs. predicted of PFOA removal; **Figure 1b:** Actual vs. predicted of PFOS removal

The data were also investigated to assess the normality of the residuals, as depicted in Figure 2a and Figure 2b for PFOA and PFOS, respectively. In these plots, the residual values are dispersed randomly across the lower and upper sections of the normal distribution line, closely resembling the line. This observation indicates that the residuals exhibit a normal distribution pattern, supporting the reliability of the analysis.

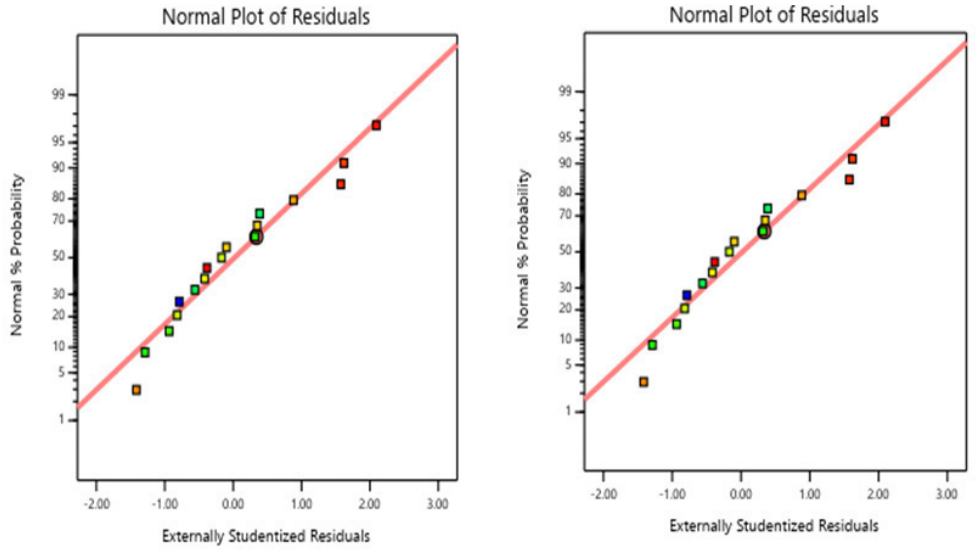

Figure 2a: Normal probability plot of studentized residual PFOA; **Figure 2b:** Normal probability plot of studentized residual PFOS

The Effects of Operating Parameters on the Removal Efficiency of PFOA

The impact values of the factors were determined using Minitab Statistic Software, enabling the generation of contour plots (Figure 3a, 3b, 3c, 3d, 3e, and 3f) based on the developed quadratic and 2FI model for PFOA and PFOS, respectively.

Figure 3a, illustrates the interaction between the initial concentration and the initial pH at an initial inoculum of 0.165 for sample containing PFOA. Notably, the highest removal efficiency, exceeding 90% was achieved within a pH range of 4 to 5 along with a dosage of 80000 ng/L and above. Conversely, the lowest removal efficiency, less than 60% occurred within a pH range of 8.8 to 9, with dosage ranging from 6000 ng/L to 90000 ng/L. Conversely for PFOS-containing samples the interaction between the initial concentration and initial pH at an initial inoculum of 0.185 indicated the highest remo-

val efficiency of more than 65%, within a pH range of 4 to 5.5 and dosages ranging from 0 ng/L to 2000 ng/L. However, the lowest removal efficiency of less than 40%, occurred at higher pH levels. It is evident that lower pH levels were associated with better removal efficiency, as represented in figure 3b.

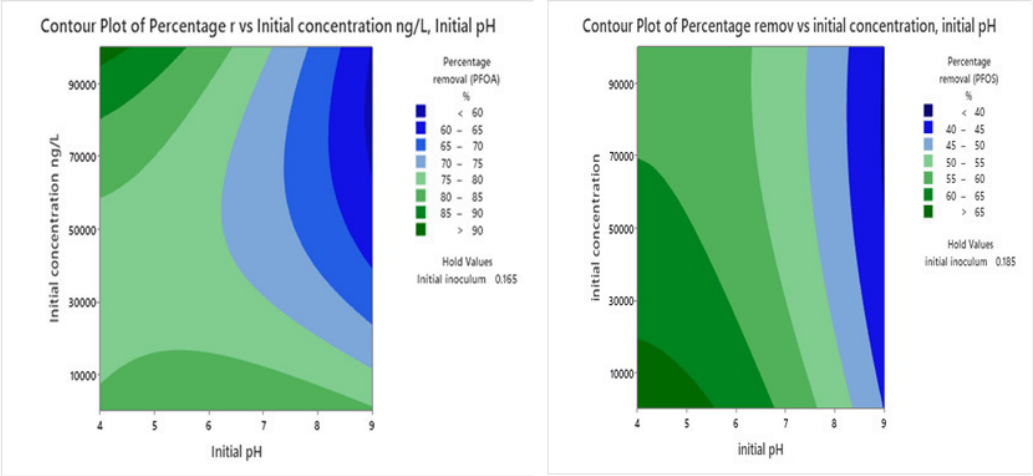

Figure 3a: Contour plot showing the interactive effect between the initial concentration and initial pH for PFOA.

Figure 3b: Contour plot showing the interactive effect between the initial concentration and initial pH for PFOS.

Figure 3c, shows the interactive effect between the initial concentration and the initial inoculum on the PFOA removal efficiency at an initial pH of 6.5. From the diagram, the highest removal efficiency exceeding 87.5% was achieved with a dosage of less than 10000 ng/L and an initial inoculum of 0.25. conversely, a removal efficiency of less than 75.0% was achieved with dosage ranging from 29000 ng/L to 80000 ng/L and inoculum concentration ranging from 0.05 to 0.20. It was reported that, the highest removal efficiency was achieved with a higher initial inoculum and a lower dosage of pollutants.

Figure 3d shows the interaction between initial concentration and initial inoculum on PFOS removal efficiency at an initial pH of 6.5. From the diagram, the highest removal efficiency exceeding 62.5% was achieved when the dosage ranged from 0 ng/L to 2000 ng/L and the inoculum concentration was 0.25 and above. Conversely, the removal efficiency of less than 50% was achieved at a higher dosage of 7000 ng/L and a lower inoculum concentration ranging from 0.05 to 0.10.

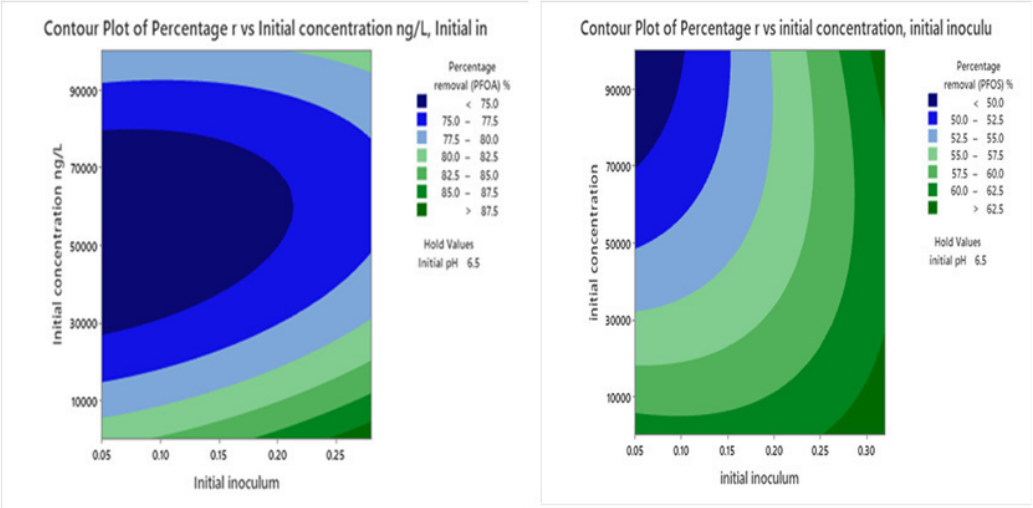

Figure 3c: Contour plot showing the interactive effect between the initial concentration and initial inoculum for PFOA; **Figure 3d:** Contour plot showing the interactive effect between the initial concentration and initial inoculum for PFOS.

Figure 3e illustrates the interaction between initial inoculum and initial pH on the removal efficiency of PFOA at an initial concentration of 50005 ng/L. From the diagram the highest removal efficiency exceeding 85%, was observed within a pH range of 4 to 4.5 and an initial inoculum range of 0.05 to 0.055. However, the lowest removal percentage, less than 55%, was achieved within a pH range of 8.6 to 9 and an initial inoculum range of 0.05 to 0.055. As it is shown on the diagram, the highest removal efficiency occurred at a lower pH, while the lowest occurred at a higher pH, with the same inoculum concentration. This highlights the influence of pH on PFOA removal efficiency, with lower pH values resulting in higher removal efficiency and higher pH values leading to lower removal efficiency. Figure 3f shows the interaction between initial inoculum and initial pH on PFOS removal efficiency at an initial concentration of 50005 ng/L. From the diagram, the highest removal efficiency exceeding 60% was observed at a lower pH range of 4 to 4.5 and the initial inoculum range of 0.05 to 0.22. On the other hand, the lowest removal percentage of less than 30% was achieved within a pH range of 6.7 to 9 and the initial inoculum range of 0.05 to 0.25.

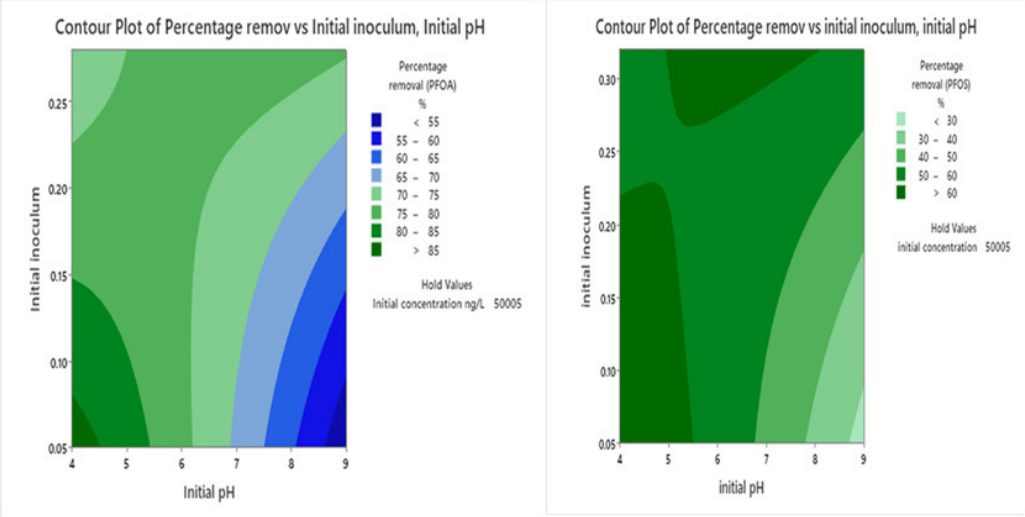

Figure 3e: Contour plot showing the interactive effect between the initial inoculum and initial pH for PFOA. **Figure 3f:** Contour plot showing the interactive effect between the initial inoculum and initial pH for PFOS.

Numeric Optimization

The model was used to determine optimal conditions for the removal of PFOA and PFOS. A comprehensive analysis of the percentage removal achieved in the 17 experimental runs by the Box–Behnken indicates that the residual behaviour adheres to a normal distribution, a pivotal assumption for assessing statistical modelling as shown in Table 5 and Table 6. The values derived from the predictive quadratic and 2FI model demonstrate good agreement with the experimental values, indicating a satisfactory correlation between them. Hence, the developed model is suitable for predicting the efficiency of PFOA and PFOS removal from wastewater utilizing activated sludge microbiome.

The primary aim of optimizing the process was to identify the optimum operational conditions to achieve maximum removal of PFOA and PFOS from wastewater. Employing the desirability function methodology, desirability ramps were developed from optimal points via numerical optimization for both PFOA and PFOS, as depicted in Figure 4 and Figure 5, respectively. Optimization involved setting goals such as none, maximum, minimum, target, or in range for the variables and response, which were then combined into a single desirability function. By utilizing numerical optimization in the Design Expert software, desirability scores of 0.99 and 0.96 were achieved for PFOA and PFOS, respectively. As illustrated in Figure 4 and Figure 5, these figures depict the range of desirability and illustrate the conditions of the optimization process.

The optimized conditions for PFOS removal using activated sludge microbiome were determined at an initial pH 4, an initial inoculum of 0.05, and an initial concentration of 50005 ng/L (Figure 4). In contrast, for PFOA removal, the optimized conditions comprised an initial pH 4.57, an initial in-

oculum 0.103, and an initial concentration of 97497 ng/L (Figure 5). According to the experimental assessment conducted under these optimum conditions, guided by the model, yielded removal efficiencies of 71.9% and 92.8 % for PFOS and PFOA, respectively. Notably, the verification tests closely aligned with the predicted response, validating the acceptability of the presented BBD model for PFOA and PFOS removal using activated sludge microbiome.

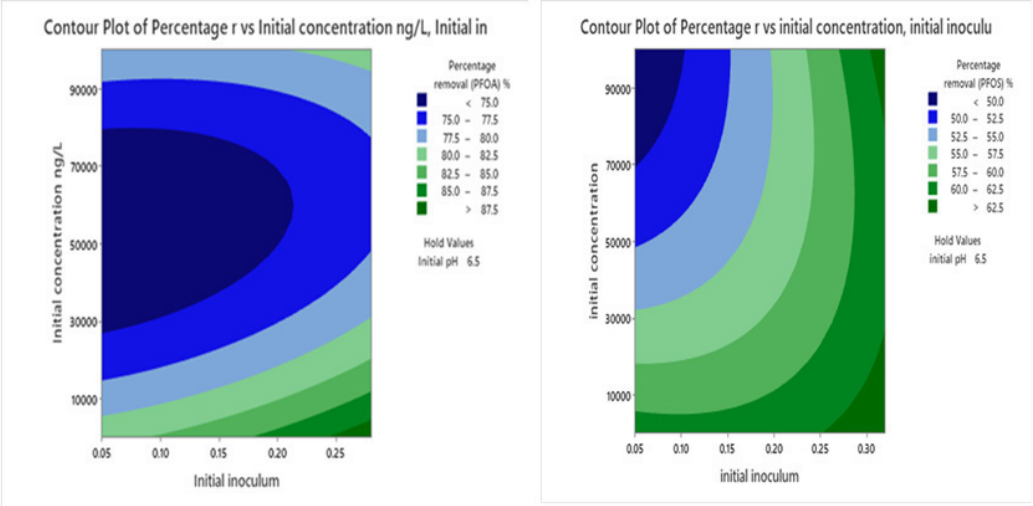

Figure 4: Desirability ramp of optimization for PFOS using activated sludge microbiome

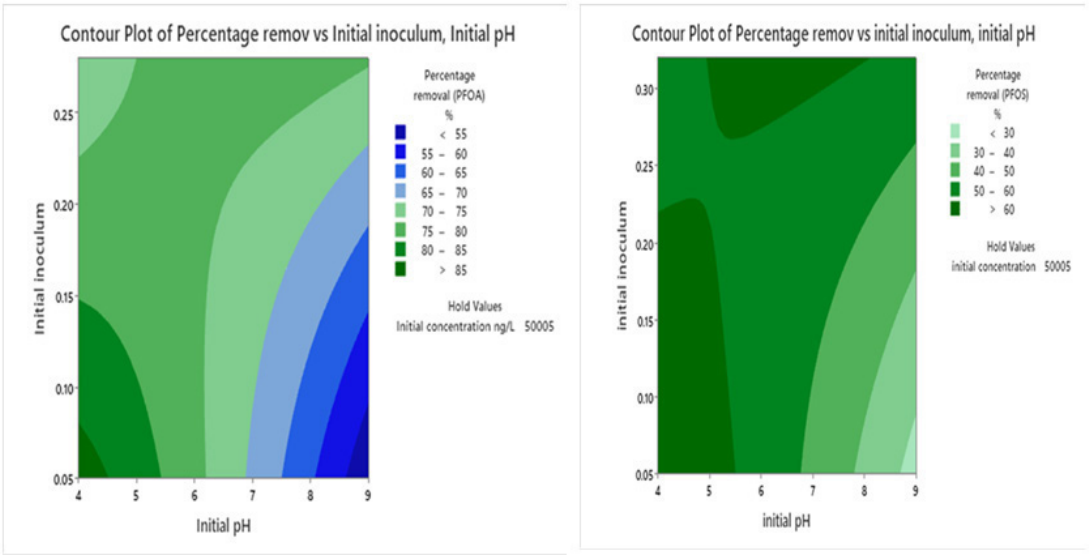

Figure 5: Desirability ramp of optimization for PFOA using activated sludge microbiome

Discussion

It is evident that the increasing use of PFASs have increased their loading in wastewater treatment plants. As a result of this increasing production and utilisation, it becomes crucial to assess the ability of the microbial communities present in wastewater to remove these pollutants to produce effluents of high quality [35]. This study focused on optimizing the biodegradation of PFOA and PFOS by the activated sludge microbiome using the BBD model. The optimization of individual response was conducted to achieve maximum removal of PFOA and PFOS based on the developed mathematical equations. Other conditions for biological reactions, such as pH and temperature, should be optimized since the PFAS biodegradation relies on microbes that function optimally under specific survival conditions [17]. The optimal value of input process parameters in this study is given in Table 5 and Table 6, demonstrating good agreement between the predicted and experimental. Notably, pH emerges as an important parameter governing the removal efficiency of PFOA and

PFOS, with lower pH levels associated with higher removal efficiencies (Fig 3a, 3b, 3c, 3d, 3e and 3f). Generally, pH can significantly influence the bioremediation of PFAS compounds through its impact on several factors such as the microbial community present, PFAS compound involved, and the environmental conditions. The interaction between the initial concentration and initial pH reported in this study revealed a higher removal rate of under acidic conditions as presented in Figure 9a and Figure 9b. Previous studies by [36] have reported the efficacy of acidic conditions in achieving high removal ratios PFOA (47.6%) and PFOS (94.7%) through coagulation. Additionally, [37], also reported that bacteria isolates have demonstrated growth capabilities in aqueous solution at a pH values of approximately 4. The Feammox process has also shown potential for PFASs biodegradation in contaminated acidic environment [38]. According to the results published on sorption capacity, it was reported that the sorption of PFAS compounds decreases with increased pH values and temperature [39]. [40] also reported that apart from bacterial composition, pH is also important when determining the degradation rate of PFAS compounds. A study by [41], reported that heterotrophic acidophilic microorganisms are an interesting potential for the bioremediation of acidic environments that contain both heavy metals and organic compounds, such as industrial wastewater effluents or oil-polluted acidic drainage waters from metal and coal mining. They further reported that a mixed culture, including a fungus, a yeast, and several bacteria, successfully metabolized about 27% of supplied naphthalene after 1 week at pH 3. In accordance with the results from this study, a lower removal efficiency of 48.1% was reported in a study conducted by [14] on the bioremediation of PFOA by Pseudomonas parafulva YAB1 at a neutral pH of 7. However, [11] reported a removal efficiency of up to 67% for PFOS by Pseudomonas aeruginosa strain HJ4 at a neutral pH of 7. Some studies suggest that certain microbial species responsible for PFAS biodegradation may have optimal pH ranges for their activity, implying that pH levels can affect the activity and growth of microorganisms involved in PFAS degradation. For example, [42] reported that acidic condition have a significant effect on the activity of Acidimicrobiaceae sp. strain A6, with an optimal pH ranging from 4 to 4.5. The explanation for this is that acidic conditions can promote the growth of specific microbial populations that enhanced PFAS degradation capabilities [14]. Le and coworkers also reported that pH level can influence the speciation of PFAS compounds in a solution. For example, at lower pH levels, PFAS compounds may exist in protonated forms, which can affect their solubility, mobility, and bioavailability. Consequently, this can impact their susceptibility to microbial degradation or other remediation processes [43,44]. Furthermore, studies have reported that PFASs removal efficiencies have been reported to vary significantly with change in pH, with optimal removal achieved under acid pH values [7,16,45]. Bacteria generally have an isoelectric point pH range of 2-5, where the cell surface carries no net charge [46]. However, when the solution pH surpasses this range, the bacterial surface becomes net-negatively charged, and conversely when pH falls below this range. A study by [47] utilized pH values within this critical range in their experiments, as changes in pH can significantly influence the net charge of the bacterial surface. At lower pH levels, there was a higher proportion of positively charged sites on the bacterial surface, such as amine groups [48]. These charged sites are more likely to interact with PFAS molecules, leading to increased sorption of PFAS onto bacterial surfaces.

Studies have shown that higher inoculum concentrations may lead to a higher removal efficiency of PFAS compounds, due to increased microbial activity. However, excessively high concentrations of PFAS may overwhelm treatment systems and inhibit microbial activity, leading in reduced removal efficiency [50]. Therefore, understanding the optimal inoculum concentration for PFASs removal is crucial for designing effective treatment strategies and ensuring environmental protection. Different bacterial species may have varying abilities to degrade PFASs. Therefore, the composition of the bacteria inoculum can also influence removal efficiency [13]. Studies often explore the dynamics of microbial communities under different inoculum concentrations to understand which species are most effective at PFASs degradation. In this study, it was reported that, the highest removal efficiency was achieved with a higher initial inoculum and a lower dosage of the pollutants (Figure 3c and Figure 3d). Some authors reported that biodegradation with specific bacteria are employed [11] reported that Pseudomonas aeruginosa can degrade PFOS up to 67% at a higher concentration ranging between 1400 and 1800 μg/L after 48 h incubation. The authors further stated that this degradation did not result in complete mineralization because the fluoride ion was not detected as a final product, rather perfluorobutanoic acid (PFBS) and perfluorohexanoic acid (PFHxS) were detected as metabolites of degradation. From the diagram in Figure 9c and 9d, the highest remo-

val efficiency exceeding 87.5% was achieved with a dosage of less than 10000 ng/L and an initial inoculum of 0.25. conversely, a removal efficiency of less than 75.0% was achieved with dosage ranging from 29000 ng/L to 80000 ng/L and inoculum concentration ranging from 0.05 to 0.20. It was reported that, the highest removal efficiency was achieved with a higher initial inoculum and a lower dosage of pollutants. There is a typical optimal range of bacteria inoculum concentration that is crucial for efficient removal of PFASs from wastewater. Too low a concentration may hinder microbial activity, while excessively high concentrations can lead to competition among bacteria for resources, reducing overall efficiency [51,52]. A higher inoculum concentration can often improve the biodegradation of PFASs in wastewater, particularly during aerobic treatment processes where microbial activity plays a crucial role. The impact of bacteria inoculum concentration on PFASs removal from wastewater treatment is a significant aspect of environmental remediation research. Therefore, optimizing bacteria inoculum concentration is essential for effective environmental remediation efforts targeting PFASs [13,49].

Duplicate confirmatory studies were carried out to validate these parameters (Figure 4 and Figure 5). A removal efficiency of 71.9% for PFOS was achieved at the following optimum conditions: an initial of pH 4, an initial inoculum of 0.05 and an initial concentration of 50005 ng/L (Figure 4). Likewise, a removal efficiency of 92.8% was achieved at the following optimum conditions: an initial pH of 4.47, an initial inoculum of 0.103 and initial concentration of 97497 ng/L (Figure 5). These results prove that PFOA and PFOS can be successfully removed from polluted environment using the microbiota from Zeekoegaat WWTP. Furthermore, the results highlight the realistic utility of Response Surface Methodology (RSM) as an optimal experimental approach for evaluating the removal efficiency of PFASs utilizing the activated sludge microbiome.

Conclusion

The Box-Behnken design in this study was used to enables the development of mathematical models to predict the removal of PFOA and PFOS utilizing the activated sludge microbiome. The results revealed that the microbiome community from the activated sludge could be used for the removal of PFOA and PFOS from aqueous solutions. Based on the experiments, many parameters affected how well our contaminants were removed. We summed up by saying:

(1) At initial inoculum levels of 0.165 for PFOA and 0.185 for PFOS. Sample containing PFOA attained a maximum removal rate of 90%, while sample containing PFOS reached 65% removal. Optimal removal occurred within a lower pH range of 4 to 5.5, indicating a direct correlation between lower pH levels and pollutants removal efficiency.

(2) At an initial pH of 6.5 for both PFOA and PFOS, maximum removal rate of 87.5% was achieved in sample containing PFOA, while 75 % removal was achieved in sample containing PFOS. Optimal removal occurred within an initial inoculum ranged of 0.05 to 0.25.

(3) At an initial concentration of 50005 ng/L for both PFOA and PFOS. Sample containing PFOA attained a maximum removal of 85%, while a percentage removal of 60% was attained in sample containing PFOS. Optimal removal occurred within an acidic pH range of 4 to 4.5 and an inoculum range of 0.05 to 0.20.

The desired removal of PFOA and PFOS can be achieved by using the predicted conditions using the BBD model. The removal efficiency of the targeted pollutants in the present study was sensitive to the pH and inoculum concentration. The high correlation between the experimental and predicted values is indicated by reported R^2 value of 0.89 and 0.79 for PFOA and PFOS, respectively. In general, sample containing PFOA consistently demonstrated higher removal efficiency as compared to those containing PFOS.

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Author's Contributions

Conceptualisation: MNBM; Data curation: MGK; Formal analysis: MGK and JOO; Funding acquisition: MNBM; Investigation: MGM; Methodology: MGK and MNBM; Project administration: MNBM; Resources: MNBM; Software: MGM

and MNBM; Supervision: MNBM and WAA; Validation: MGM; Visualisation: MGK; Writing - original draft: MGM and MNBM; Writing - review & editing: WAA and MNBM.

Ethics Approval

This study does not involve direct human or clinical isolates. However, for the collection of activated sludge sample at Zeekoegaat wastewater treatment plant permission was obtained from the plant manager.

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