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NH_3 and COD removal from wastewater using biological process: kinetic with optimization studies

Ayusman Mohanty, Sanak Ray, Asheesh Kumar Yadav, G. Roy Chaudhury*

CSIR-Institute of Minerals and Materials Technology, Bhubaneswar 751013, India
Tel. +91 9338810225; +91 9437115485; +91 674 2379306 (O); +91 674 2379540 (O); Fax: +91 674 2581637; email: gr_chaudhury@yahoo.com

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ABSTRACT

Studies on heterotrophic biomass conversion (HBC) process were carried out for the removal of N–NH_3 and organic carbon from wastewater. Ammonium sulfate and glucose were used as nitrogen and organic sources, respectively. A range of parameters were studied such as time, concentration variations of N–NH_3, and organic nutrients keeping the biomass (total volatile suspended solids, TVSS) concentration invariable in all the cases. The kinetics followed dual rates, i.e. an initial faster phase, followed by the slower one. The rates of N–NH_3, and chemical oxygen demand (COD) removal depended on their initial concentrations. The consumption of N–NH_3 and COD followed first order kinetics. The unified rate equation was also established. Two other kinetic models, such as Monod and diffusion, were studied. The pH during the HBC process showed a decreasing trend. Other parameters studied were: N–NO_3^-, N_2O, N–NO_2^-, and DO. A part of N–NH_3 utilized for emission of N_2O may be due to heterotrophic nitrification (HN). Statistical studies were carried out such as analyses of variance (ANOVA), multi linear regression analysis and principal component analysis (MLRA), and pricipal component analsis (PCA). Optimization studies were carried out using response surface modeling (RSM) to maximize the removal efficiency of N–NH_3 and COD, minimizing the N–N_2O emissions, along with maximizing the TVSS production simultaneously.

Keywords: Heterotrophic biomass conversion; Kinetics; Monod; Diffusion; Wastewater treatment; Optimization; Statistical technique

1. Introduction

Aquatic pollution is considered as one of the important environmental problems presently. Rapid growth in agriculture, industrialization, and urbanization leads to the generation of a huge amount of wastewater. This wastewater generally contains various forms of nitrogen and organic carbon, which act as pollutants. The nitrogenous wastes released to aquatic bodies leads to eutrophication [1]. The organic pollutants in water bodies usually decrease dissolved oxygen (DO) leading to the death of aquatic animals and plants.

Various methods are adopted for nitrogen removal from wastewater, but the most economical one is the biological treatment. Generally nitrifying and denitrifying bacteria are used in biological treatment [1–3].

*Corresponding author.

Emission of N_2O has been observed during wastewater treatment [2,4], which is a significant green house gas (GHG) [1,5,6]. It initiates global temperature swell and causes ozone depletion [7–9]. Denitrifying and nitrifying bacteria are reducing and oxidizing bacteria, respectively. The former reduces NO_3^- to N_2 whereas the latter oxidizes NH_3 to NO_3^- [2,10]. Therefore, the total process goes through two steps: anaerobic and aerobic [2,11]. To resolve the dilemma, other processes, such as anaerobic ammonium oxidation (ANAMOX) as well as heterotrophic biomass conversion (HBC) have been developed. NH_3 is converted to N_2 through an intermediate N_2O in the ANAMOX process, whereas HBC directly converts NH_3 to biomass total volatile suspended solids (TVSS), thus retaining the nitrogen values. The HBC process requires organic carbon source to convert NH_3 to TVSS. The HBC process is mostly carried out by the facultative heterotrophs under aerobic conditions and they may go for denitrification under anaerobic conditions. The reaction kinetics depends on various factors, such as pH, alkalinity, temperature, oxygen, and NH_3 concentrations [12]. The HBC process consumes alkali and releases CO_2 as a byproduct, which reduces the pH [12]. Oxygen demand is slightly higher in the process, which can be achieved through proper aeration [12]. Since there is no N_2O emission, the process can be considered as environment friendly. Customarily, in HBC process TVSS production is 40 times greater than the TVSS generated in nitrification process [12], which can be used as biofertilizer. Conversion of NH_3 to NO_3^- by heterotrophic nitrification (HN) is reported to be carried out by heterotrophic bacteria under aerobic conditions releasing NO_3^- as the final product through N_2O as an intermediate product [10]. Although the substrate, intermediates, and products of heterotrophic and autotrophic nitrification are the same, the enzymes of the two processes have been shown to differ from each other [10]. The pathway of heterotrophs resembled that of the autotrophs in HN. Ammonia is oxidized to hydroxylamine by ammonia monooxygenase and then hydroxylamine is oxidized to nitrite by hydroxylamine oxidase, which is a key reaction. Co-respiration is an important mechanism in aerobic denitrification, where oxygen and nitrate are simultaneously used as electron acceptors [13].

The pH normally goes below 7 in HN course, initiating higher N_2O emission [10,14]. It may be prevented by maintaining the pH at nearly neutral level. Organic carbon is used by nitrifiers as a source of energy. The substrates, intermediates and products are the same for both heterotrophic as well as autotrophic nitrifiers. Generally, the HN process produces a little N_2O but it gets maximized under circumstances like high DO, low pH and, availability of organic source [10]. Some bacterial species like *Pseudomonas* and *Alcaligenes*, are termed as heterotrophic nitrifiers [15]. Yet, ample understanding of HN progression based on the detailed physiological as well as parametric studies in batch and continuous cultures is desirable.

The HBC process is an intricate one; hence, an in-depth analysis is required to link it to various process parameters. Keeping the above in view, attempts have been made to study the kinetics, establishment of rate equation, evaluation of rate determining steps, statistical construal of results like analyses of variance (ANOVA), multi-linear regression analyses (MLRA) and, principal component analyses (PCA). However, this treatment process involves emission of N_2O which in turn depends on the concentration of nutrients like chemical oxygen demand (COD) and nitrogen source along with microbial concentration. Complicacy of the total process with insufficient availability of literature in this field increases the size of this problem. Response surface modeling (RSM) technique is used to optimize the response of a multivariate system by exploring the relationships between various variables and their combined effects on response variables [16–18]. This technique is also used to reduce the number of experiments and to develop mathematical models to establish the interactions between the process variables precisely. The model was verified through experiments and tested statistically. Batch experiment studies were conducted to study the effect of variables such as nitrogen and organic source along with days. The main objective of this study is to maximize the N–NH_3 output along with COD removal efficiency, minimize the N–N_2O emission and simultaneously maximize the TVSS formation as various responses through RSM.

2. Material and methods

2.1. Sampling and enrichment

Soil sample was collected from a paddy field in Tangibanta, a rural location 20 km away from Bhubaneswar, India, at a depth of 0-15 cm using an auger [19]. The typical soil analysis showed: Na: 1.5%, Mg: 1.8%, Al: 20.5%, Si: 53.2%, K: 1.2% and Ca: 2.4%. It also contained micronutrients like carbon, nitrogen and phosphorous. The denitrifying heterotrophic bacteria were isolated from the agricultural soil using MSN (mineral, salt, and nutrient) liquid media (composition: CH_3COONa-7.86 g/L, $KH_2PO_{4–0}$.2 g/L, $(NH_4)_2SO_{4–0}$.5 g/

L, $MgSO_4.7H_2O$-0.04 g/L, and $Ca(NO_3)_2$-0.04 g/L) [1]. The isolated strains were enriched on a regular basis by re-inoculating into freshly prepared MSN media to enrich the TVSS and increase their activity. The mixed consortium mainly contained species like *Pseudomonas aeruginosa, Proteiniphilum acetatigenes* and *Alcaligenes faecalis*. Identification of the species was carried out by 16S rDNA based method at the Indian Institute of Technology, Roorkee. Each experiment was carried out in duplicate and the average value was taken for the interpretation of results. The variation of duplicate rate was within a range of ± 5%.

2.2. Experimental setup

As the mixed bacterial consortium is facultative in nature, incubation studies were carried under aerobic conditions, which are favorable to carryout HBC and HN process. The concentration of $N–NH_3$ and organic carbon were varied to study the HBC kinetics. Ammonium sulfate [$(NH_4)_2SO_4$] and glucose ($C_6H_{12}O_6$) were used as nitrogen and organic sources. The incubation experiments were carried out in 100 ml of solution (synthetic wastewater) containing 90 ml MSN media excluding the nitrogen and organic source, 9 ml mixed consortium and 1 ml of various concentrations of nitrogen source using incubation bottles (Borosil, 250 ml). The nitrogen and organic source concentrations were ranged from 50 to 250 mg/L and from 500 to 1,500 mg/L, respectively. The incubation bottles along with samples were kept in Julabo SW-22 shaking incubators at 35˚C to maintain aerobic conditions. The gas and liquid samples were drawn at regular intervals (12 h) using hypodermics syringe for analyzing N_2O along with various water parameters. The gas samples bottles were covered with air tight rubber caps for one hour. Seventy milligram inoculums were used for each bottle initially in incubation studies. The entire sets of experiments were carried out for three days.

2.3. Gas sampling and analysis

Air samples were drawn through disposable syringe at 12-hours interval. A gas chromatograph (GC) of make (Shimadzu AA30) with electron capture detector was used to analyze N_2O concentration. The GC is equipped with auto gas samplers, semi-micro columns and appropriate software to process the acquired data. The GC was regularly standardized using NIST primary standard gases. N_2O in solution was determined by drawing a known amount of solution by a hypodermic needle and introduced to a reactor under vacuum. After 5 min, the N_2O stripped was drawn and analyzed in GC as explained previously. The dissolved N_2O in solution was estimated using Bunsen absorption coefficient as shown below in Eq. (1) [20].

$$Y = x \times \alpha \quad \text{(Solution volume/head space volume)} \tag{1}$$

where $\alpha = 0.485$, x is mass in head space, and y is mass in solution.

2.4. Water sample analysis

Parameters like Nitrate nitrogen ($NO_3–N$), Nitrite nitrogen ($N–NO_2$), Ammonia nitrogen ($N–NH_3$), DO and COD were analyzed following standard methods [19,21]. The pH of the samples was measured in 12 h interval using EUTECH-pH 1,500 meter. Analysis of TVSS was performed following standard methods [21].

2.5. Kinetic study

Various approaches like evaluation of rate equation, mass transfer coefficient, and Monod were carried out to determine the reaction kinetics for different variables like variation of $(NH_4)_2SO_4$ and $C_6H_{12}O_6$ using Microsoft office excel 2007 program.

2.6. Statistical analysis

2.6.1. ANOVA

ANOVA studies were carried out using Microsoft office excel 2007 program.

2.6.2. Multivariate statistical analyses

The incubation data were subjected to multivariate statistical analysis to evaluate the inference of various incubation parameters on the nutrients removal rates. MLRA have been previously utilized [22] to determine the significance of specific parameters among datasets. MLRA was conducted using the step-wise forward integration method using SPSS-10.

2.6.3. PCA

The incubation data were subjected to PCA for evaluating the influence of various incubation parameters on the HBC and HN rates. PCA was conducted using SPSS-10 previously to determine significance of various parameters [22]. Eigen values were used to

determine the percentage of variance as well as the cumulative percentage of variances in PCA. A varimax rotation of different varifactors with factor loading was calculated using Eigen values > 1 and sorted by the results having value > 0.5 to have $p < 0.01$.

2.6.4. RSM (optimization)

The incubation studies were carried out by statistically designed experiments [16–18,23]. The principal steps are determination of response variables, factors and factor level, choice of experimental design and statistical analysis of data. Experiments were carried out by using ammonium sulfate and glucose as nitrogen and organic source for this purpose. Design-Expert 8 was used for this work.

3. Results and discussion

3.1. Variation of ammonium sulfate as nitrogen source

A series of experiments were carried out by varying ammonium sulfate ($(NH_4)_2SO_4$) concentration from 50 to 250 mg/L N–NH_3 keeping glucose ($C_6H_{12}O_6$) at 5 g/L. Thus, the ammonium concentrations can be considered as a limiting factor in the present studies [12].

Fig. 1 shows the typical curve of biomass conversion of N–NH_3 (250 mg/l) along with changes in other parameters such as pH, TVSS, DO, COD, nitrogenous compound like N–NO_2, N–NO_3, and N–N_2O. Similar curves are obtained for other $(NH_4)_2SO_4$ concentrations and so are not shown. In all the cases, the removal efficiency for nitrogen can be divided into two parts, i.e. initial faster rate followed by the slower one. The faster rate accounted for $\geqslant 80\%$ of the total removal of $(NH_4)_2SO_4$. The faster kinetics lasted up to 40 h and thereafter followed by the slower one. The initial faster rate may be due to easy availability of the substrate and gradual depletion of the substrate with time resulting in slower kinetics. Keeping in view of the above observations, the subsequent nutrient removal processes were limited to 60 h.

The pH during the reaction showed gradual decrease as the reaction is acid producing one [12] as shown below in Eq. (2).

$$NH_4^+ + 1.18\ C_6H_{12}O_6 + HCO_3^- + 2.06\ O_2 \rightarrow C_5H_7O_2N + 6.06\ H_2O + 3.07\ CO_2 \quad (2)$$

The DO concentration always remained at around 7 during the entire incubation period, which suggests good aerobic condition for carrying out the reaction. The theoretical O_2 requirement for converting 1 g of N–NH_4^+ to TVSS was calculated to be 4.71 g and this amount of O_2 cannot be provided considering the DO value unless the same would have been diffused during aeration. The COD concentration progressively decreased with time as $(NH_4)_2SO_4$ used it up while converting to TVSS vide Eq. (2). On the contrary, the TVSS concentration increased with time. Other nitrogen compounds like nitrite (NO_2^-) and nitrate (NO_3^-) showed some interesting results. The NO_2^- and NO_3^- concentration also increased with time. The HBC does not involve in the formation of the said nitrogenous compounds. The formation of these two compounds during the reaction unequivocally suggests HN [10,24]. During HN, the ammonia (NH_3) is converted finally to NO_3^-. It passes through various intermediate

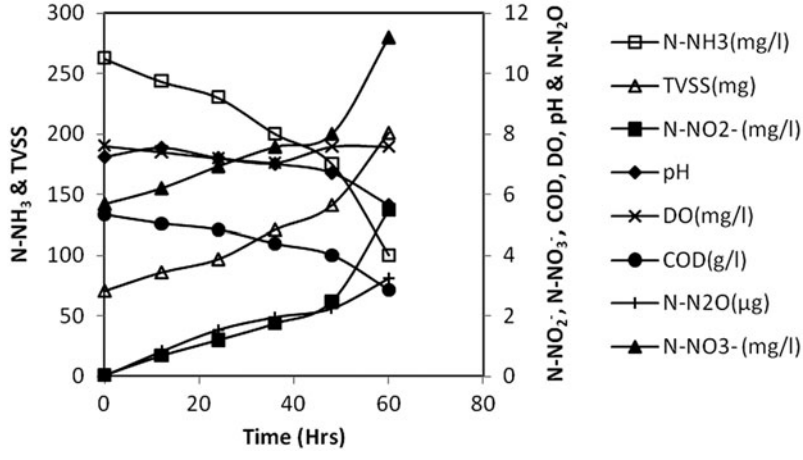

Fig. 1. Behavior of variables like N–NH_3, TVSS, pH, DO, COD, N–NO_2^-, N–NO_3^-, N–N_2O and time during incubation studies. (Initial conditions: ($(NH_4)_2SO_4$)—250 mg/L N–NH_3, $C_6H_{12}O_6$- 5 g/L, Temp- 35°C, pH-7).

Table 1
Determination of rates and dependence factor

Rate-ammonium sulphate variation								
Dependence factor								
$N–NH_3$ (mg N/L)	$N–N_2O$ (μg N/h)	$N–NO_3^-$ (mg/L N/h)	$N–NO_2^-$ (mg/L N/h)	$N–NH_3$ (mg/L N/h)	COD ((mg/L)/h)	TVSS (mg/h)	R^2	$n1$
50	0.05	0.08	0.13	1.26	19.16	1.02	0.85	0.23
100	0.07	0.13	0.14	1.33	20.45	1.07		
150	0.09	0.15	0.16	1.42	21.59	1.15		
200	0.11	0.21	0.19	1.68	25.51	1.36		
250	0.14	0.23	0.23	1.84	27.89	1.48		
Rate-glucose variation								
$C_6H_{12}O_6$ (mg/L)	$N–N_2O$ (μg N/h)	$N–NO_3^-$ (mg/L N/h)	$N–NO_2^-$ (mg/L N/h)	$N–NH_3$ (mg/L N/h)	COD ((mg/L)/h)	TVSS (mg/h)	R^2	$n2$
500	0.02	0.01	0.01	0.51	7.70	0.41	0.98	0.65
750	0.02	0.02	0.01	0.58	9.26	0.47		
1,000	0.03	0.05	0.02	0.75	11.42	0.61		
1,250	0.04	0.06	0.02	0.95	14.39	0.77		
1,500	0.05	0.07	0.03	0.99	15.05	0.80		

products like hydroxyl amine, NO_2^-, and N_2O gas during the formation of the NO_3^- [10]. We observed that N_2O concentration, both in the liquid and gaseous phases, depends on NO_2^- concentration. The increase of nitrite concentration in the solution showed higher release of N_2O gas [25]. The concentration of N_2O in the liquid was less compared to air. The reaction rates for $N–NH_3$, $N–NO_2^-$, $N–N_2O^-$, $N–NO_3^-$, COD and TVSS are shown (Table 1) for all the incubation studies. An attempt was made to balance the nitrogen using the analyzed results. The utilization of nitrogen for $N–N_2O$ was marginal, i.e. < 0.1%. Hence, it can be concluded that the incubation studies do not involve appreciable emission of GHG.

3.2. Variation of glucose as carbon source

A series of experiments were carried out by varying the $C_6H_{12}O_6$ concentration from 500 to 1,500 mg/L keeping the $(NH_4)_2SO_4$ concentration constant at 100 mg/L $N–NH_3$. These set of experiments were carried out under COD limiting conditions. The parameters like COD, $N–NH_3$, $N–NO_2^-$, $N–NO_3^-$, $N–N_2O$, pH, DO and TVSS were measured at regular intervals following procedure followed earlier, the results of which are shown in Fig. 2. $N–NH_3$ depletion followed dual rate: an initial faster rate followed by the slower one. The DO in all cases was ⩾7 indicating proper aerobic conditions during the incubation studies.

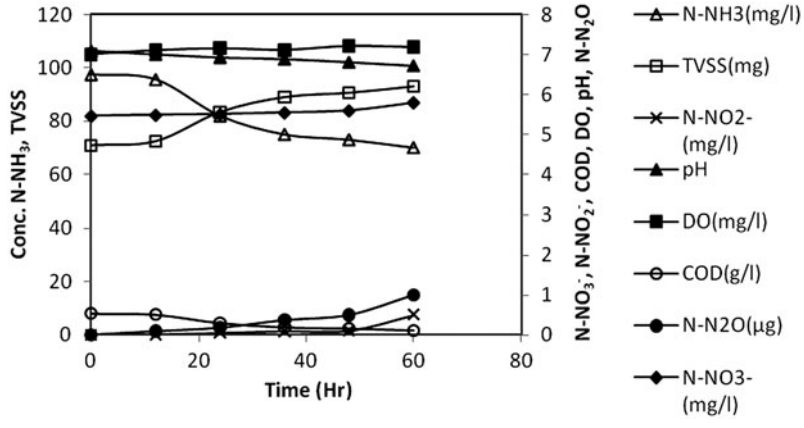

Fig. 2. Behavior of variables like $N–NH_3$, TVSS, pH, DO, COD, $N–NO_2^-$, $N–NO_3^-$, $N–N_2O$ and time during incubation studies. (Initial conditions: $C_6H_{12}O_6$- 500 mg/L, $((NH_4)_2SO_4)$—100 mg/L $N–NH_3$, Temp-35°C, pH-7).

The pH progressively decreased with time. The N–NH_3 reaction rate showed a positive correlation with initial COD concentration. The rate of formation of NO_3^- and NO_2^- was calculated to be less compared to the earlier case as shown in Table 1, this may be due to lower NO_2^- reaction rate. The rate of formation of N_2O was less due to lower formation of NO_2^-. The TVSS formation rate is shown in Table 1. The calculated value for utilization nitrogen for the formation of N–N_2O was < 0.15%.

3.3. Kinetic consideration

3.3.1. Evaluation of rate equation

An attempt was made to evaluate the order of reaction for different variables like variation of $(NH_4)_2SO_4$ as well as $C_6H_{12}O_6$. Two different orders of reactions were considered for this purpose, such as first and second order. A reaction would be of first order if the plot between log (concentration) vs. time would give a straight line and the slope would give a specific reaction rate. Similarly, for the second order, a plot between the reciprocal of concentration vs. time would give a straight line and specific reaction rate would be obtained from the slope. The results are shown in Table 1. By considering the coefficient of determination values, it can be concluded that the reaction rate in the both cases followed first order kinetics. Since the reaction depends on the concentrations of ammonium sulfate as well as glucose, the reaction may be considered as pseudo-first order. The HBC depends on the variables like ammonium sulfate and glucose concentrations. For that reason, the rate of equation can be written as:

$$\text{Rate} = -\mathrm{d}c/\mathrm{d}t = k(\text{ammonium sulphate})^{n1}(\text{glucose})^{n2} \quad (3)$$

where c = concentration of constituents determining the HBC and n is the order of reaction [26]. By converting the equation to logarithmic form, we get:

$$\log(R) = \log k + n_1 \times \log(\text{ammonium sulfate}) + n_2 \log(\text{glucose}) \quad (4)$$

Experimental data are arranged to fit into Eq. (4) in order to determine the order of reaction. For this purpose only one parameter was varied at a time keeping the other parameter constant. The n values were found to be 0.23 and 0.65 for ammonium sulfate and glucose, respectively. Therefore, the rate equation can be written as:

$$\text{Rate} = -\mathrm{d}c/\mathrm{d}t = k(\text{ammonium sulfate})^{0.23}(\text{glucose})^{0.65} \quad (5)$$

3.3.2. Evaluation of mass transfer coefficient

The substrate utilization kinetics in a HBC reaction by bio-film can be explained through diffusion model [27]. If the reaction is limited by diffusion then four different situations may arise in the reactor [27] as shown below:

(a) The deep bio-film and low substrate concentration scenario may result in pseudo first-order kinetics for the substrate depletion rate and would be independent of the thickness of bio-film.
(b) If the bio-film thickness is high and so also the substrate concentration; then the concentration of the substrate on the bio-film would be approaching that of the bulk. So, the reaction rate can be considered as diffusion controlled and the rate equation can be written as:

$$\mathrm{d}C_s/\mathrm{d}t = k_2(C_s)^{0.5} \quad (6)$$

where k_2 is diffusion constant

(c) If the bio-film is porous and the concentration of the substrate is minimal, then the reaction would follow a pseudo first order kinetics.
(d) If the bio-film is porous and the substrate concentration is high, then the reaction would follow zero-order kinetics with the following rate reaction:

$$\mathrm{d}C_s/\mathrm{d}t = k_3 \quad (7)$$

where k_3 is the rate constant; $\mathrm{d}C_s/\mathrm{d}t$ = rate of NH_3 depletion, t = time and K = mass transfer rate constant.

Fig. 3 shows the plot between rates of substrate depletion vs. $(C_s)^{0.5}$. The graphs show good co-linearity as the coefficient of determination in all cases is $\geqslant 0.9$, which suggests that the reaction would be diffusion controlled and thereby chances of intra-particle reaction would be minimal and may not be influencing the rate determining step. The mass transfer rate for N–NH_3 and COD depletion conditions were calculated to be 0.06 and 0.03 $(h)^{-1}$ respectively.

3.3.3. Evaluation of Monod model

In case of single-substrate limited process Monod equation as shown in Eq. (8) can be used to describe

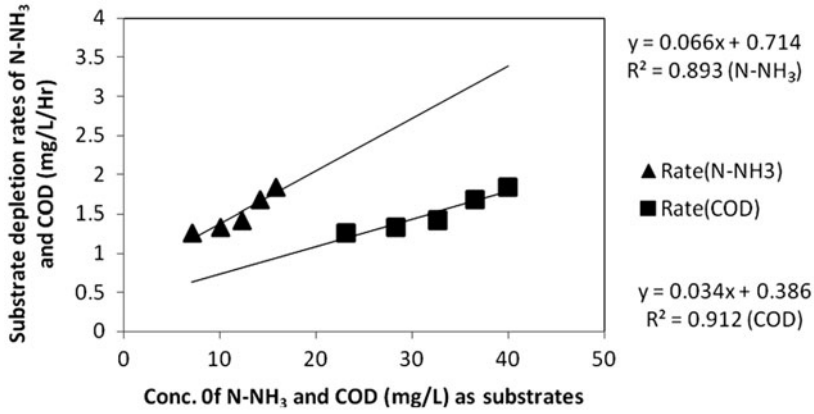

Fig. 3. Plot between rates of substrate depletion vs. concentrations for both N–NH_3 and COD.

the microbial growth and degradation of materials like ammonia and COD [11,27].

$$dC_s/dt = k_4 C_x C_s/(K_s + C_s) \quad (8)$$

where, dC_s/dt = substrate consumption rate (mg/L/h), K_s = half saturation constant (mg/L), C_s = concentration of substrate (mg/L), C_x = TVSS concentration (mg/L), k_4 = maximum specific degradation rate (mg/L/h).

The kinetic parameters like k_4 and K_s can be estimated from the slope and intercept of the straight line of Lineweaver-Burk type plots considering the C_x concentration nearly constant. In all our experiments the total volatile suspended solid (TVSS) ranged in the range 0.75–1.6 g/L. The slope and intercept values were calculated by plotting 1/rate of substrate (considering the substrate limitation only) (mg/L/h) vs. 1/concentration (mg/L).

In the present case, two different substrate limiting cases were considered, such as ammonium sulfate and glucose. Thus, two different K_s and k_4 values would be obtained as shown in Fig. 4. The k_4 and K_s values for $(NH_4)_2SO_4$ limiting case are 1.80 and 24.375, respectively. Similarly for $C_6H_{12}O_6$ limiting case the k_4 and K_s values were 28.571 and 1503.114, respectively. The data fit was good as the coefficient determination values were high. The half saturation constant in both cases are high [11] indicating that the substrate removal rate depend on the substrate concentration over a wide range.

3.4. Statistical interpretation

3.4.1. Significance level determination

Two parameters were varied in the experiments, such as the concentration of $(NH_4)_2SO_4$ and $C_6H_{12}O_6$.

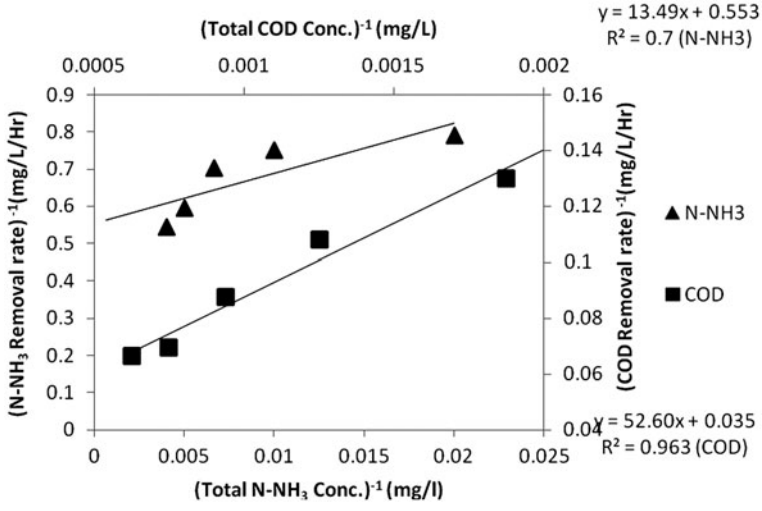

Fig. 4. Plot between 1/rate (mg/L/h) of substrate vs. 1/conc. (mg/L) for both N–NH_3 and COD.

Table 2
ANOVA for various parameters

	Variables glucose			
	F-value	*P*-value	*F*-critical	Remarks
Depletion of $N–NH_3$				
Hours	31.63	7.674E-09	2.71	Significant
$N–NH_3$	7.55	0.0007053	2.87	Significant
TVSS formation				
Hours	31.63	7.674E-09	2.71	Significant
TVSS	6.15	0.0021363	2.87	Significant
	Ammonium sulfate			
Depletion of $N–NH_3$				
Hours	74.28	3.205E-12	2.71	Significant
$N–NH_3$	363.01	2.329E-18	2.87	Significant
TVSS formation				
Hours	74.28	3.205E-12	2.71	Significant
TVSS	4.38	0.0104689	2.87	Significant

A series of experiments were carried out to find out the nutrient removal efficiency with time. Two-way ANOVA was carried out for both the parameters and the results are shown in Table 2. In all the cases the variation of time and nutrient concentrations are significant, which means that both the parameters play important roles.

3.4.2. MLRA

Multiple linear regression analysis is a statistical technique in which a single correlation is established between a dependent variable and several independent variables. In the present study, the TVSS concentration was considered as dependent variable and other parameters, such as COD, $N–NH_3$, and time, were the independent variables. The primary objective of this analysis was to use independent variables with values known to be capable of predicting the dependent variable to estimate the TVSS concentration. The reliability of this method can be evaluated based on various statistical parameters, such as the coefficient of determination (R^2), standard error, beta, significance, *F*-change, and significance of *F* change, as shown in Table 3.

Using the coefficients and constant values the theoretical TVSS concentrations in each case was determined. The theoretical values were compared with the experimental values as shown in Fig. 5(a). It

Table 3
Various statistical parameters obtained during MLRA

	R	Std. error	*F* change	Sig. *F* change
Model summary	0.88	11.84	64.33	2.71497E−18
Variable	Coefficients	Std. error	Beta	Sig.
Constant	65.106	4.719		8.09841E−20
Time	0.945	0.082	0.80	1.99725E−16
$N–NH_3$	−0.018	0.034	−0.04	0.59
COD	0.005	0.001	0.43	9.38363E−08

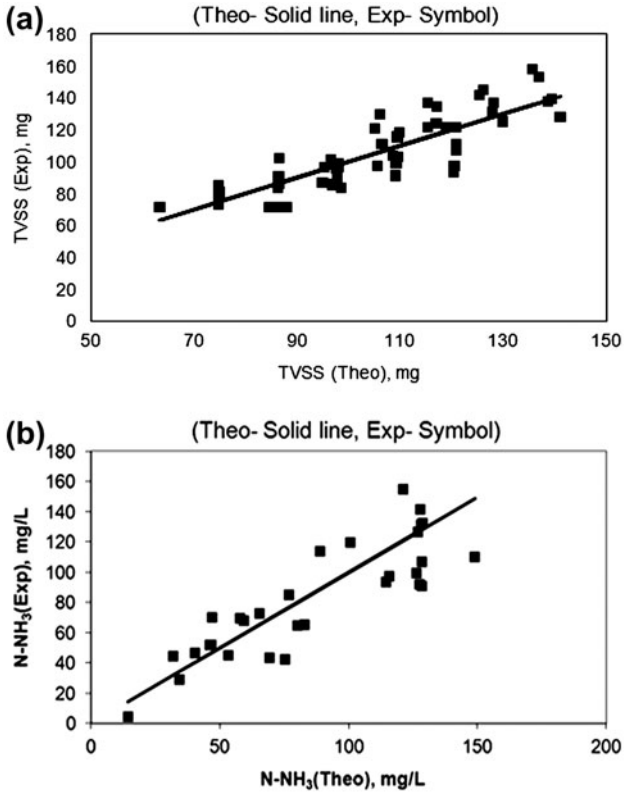

Fig. 5. Theoretical vs. experimental values of TVSS (a) and $N–NH_3$ (b) obtained from MLRA.

shows good matching between the theoretical as well as experimental values, which indicates that the theoretical TVSS concentration can be evaluated for any unknown condition within the upper and lower limit of the experimental conditions such as time and nutrient concentrations.

Apart from the above, correlation studies were also carried out for other parameters such as DO, pH, and concentrations of nitrogenous compounds such as $N–NO_3^-$, $N–N_2O$ and $N–NO_2^-$ through MLRA. The

concentrations of nitrogenous compounds were marginal and so also the change. DO and pH were marginal in all experimental cases. These factors greatly depend on the concentrations of $N–NH_3$. Consequently $N–NH_3$ was considered as dependant variable and all other above parameters as independent variables. A correlation was made between the dependant and independent variables and a comparison was done between the $N–NH_3$ theoretical and as well as experimental values. The results are shown in Fig. 5(b). It shows good matching between the theoretical as well as experimental.

3.4.3. PCA

PCA or factor analysis is a part of multivariate statistical method. It usually analyzes the intra-relationship of a large number of variable [28]. In the present case, the variables are time, $N–NO_3^-$, TVSS, COD, p^H, DO, $N–NO_3^-$, $N–NO_2^-$, and $N–NO_2^-$. The factors are nothing but the inter correlation of variables having same character or dimension. So the primary use for factor analysis is summarization and data reduction. In our present case most of the variables can be classified under two factors. Inter-correlation in a factor is obtained using correlation matrix as shown in Table 4. Similar factor analysis (PCA) have been reported by many authors [29–31]. PCA was carried out in order to determine the intra-correlationship between different variables. The correlation matrix is shown in Table 4. Time showed positive correlation with TVSS, $N–NO_3^-$, $N–NO_2^-$ and $N–N_2O$, which indicates that all these concentrations would increase with time. $N–NH_3$ showed positive correlation with COD, which is obvious as both are considered as nutrients and would be consumed by heterotrophic microorganisms, vides Eq. (2). TVSS, on the other hand, showed positive correlation with $N–NO_3^-$, $N–NO_2^-$ and $N–N_2O$. In the present case, HBC of the nutrient does not involve the production of $N–NO_3^-$, $N–NO_2^-$ and $N–N_2O$ and these can only be possible through nitrifying bacteria and therefore the positive correlation indicates that the TVSS might have contained both heterotrophic and nitrifying bacteria. NO_3^- showed positive correlation as it relates to bacterial nitrification reactions. Similarly, $N–NO_2^-$ showed positive correlation with $N–N_2O$, which suggests that the increase in of $N–NO_2^-$ would increase the $N–N_2O$ emission.

The PCA is shown in Table 4. It contains two components: Component-1 explains 45.88% with a Eigen value of 4.23. It contains variables like time, TVSS, $N–NO_3^-$, $N–NO_2^-$, and $N–N_2O$. All are positively correlated signifying that the increase of one variable would increase the other. The inclusion of $N–NO_3^-$, $N–NO_2^-$ and $N–N_2O$ means it all relates to nitrification. Therefore, Factor-I can be termed as "nitrification." The factor explained a cumulative variance 65.97% of total variance with a Eigen value of 1.7. It contains two variables such as $N–NH_3$ and COD. Both the variables play a vital role during heterotrophic nitrogen conversion to TVSS. Therefore, Factor-II can be termed as "heterotrophic biomass conversion".

Table 4
Principal component analysis

Correlation matrix

	Time	$N–NH_3$	TVSS	COD	pH	DO	$N–NO_3^-$	$N–NO_2^-$	$N–N_2O$
Time	1								
NH_3	−0.41	1							
TVSS	**0.77**	−0.18	**1**						
COD	−0.1	0.44	0.33	**1**					
pH	−0.23	0.22	−0.23	0.22	**1**				
DO	−0.2	0.04	−0.13	0.02	0.16	**1**			
$N–NO_3^-$	**0.55**	−0.01	**0.72**	0.18	−0.33	−0.1	**1**		
$N–NO_2^-$	**0.59**	−0.07	**0.74**	0.22	−0.31	−0.11	**0.98**	**1**	
$N–N_2O$	**0.75**	−0.18	**0.72**	−0.03	−0.38	−0.14	**0.84**	**0.83**	**1**

Rotated component matrix

Component	Time	$N–NH_3$	TVSS	COD	pH	DO	$N–NO_3^-$	$N–NO_2^-$	$N–N_2O$	Eigen values	Cumulative variance
1	0.75		0.89				0.93	0.94	0.89	4.24	45.88
2		0.8		0.82						1.7	65.97

3.4.4. Optimization of responses using RSM

HBC studies were carried out by varying two parameters such as concentrations of $(NH_4)_2SO_4$ and $C_6H_{12}O_6$. Samples were collected at regular intervals to analyze the product such as N–NH_3, COD, N–NO_2^-, N–N_2O, N–NO_3^- and TVSS. Among all these parameters the concentrations of N–NH_3, COD, N–N_2O, TVSS and Time were taken into consideration. Keeping the above in view, efforts were made to optimize the overall process through surface response methodology. Coding was done to reduce the range of each factor to a common scale regardless of the magnitude; the typical scheme being to set-1 as lower level, +1 as upper level and 0 as middle level with the Eq. (9) given as:

$$\text{Code} = \frac{\text{Actual value} - \text{Factor mean}}{\text{Range of factorial value}/2} \quad (9)$$

Central composite design with two labeled factorial points such as axial and center was done [18,23]. The axial point having all factors was set to zero, except one factor which had the value $\pm\alpha$. α represents the distance from the center of the designed space to an axial point. Since in this study the factors were $\leqslant 5$ the rotatable model was chosen which corresponds to the α value 1.68. The factorial points such as N–NH_3, COD and time were considered as inputs to analyze the responses like N–NH_3, N–N_2O, COD and TVSS. It also shows the design summary along with the solutions obtained during optimization. Table 5 shows the final equation in terms of coded factors and respective F values, Prob > *F* values, remarks, R^2 values and adequate precision along with model fit. The actual and calculated values for the responses are shown in Fig. 5(a) using the equation shown in Table 5.

Table 5
Design summary

Factor	Name	Units	Minimum	Maximum	Mean	Std. dev.		
A	N–NH_3	mg/L	5.57	249.43	127.5	59.91		
B	COD	g/L	0.04	6.26	3.15	1.53		
C	Time	Hrs	1.11	49.89	25.5	11.98		
Response	Name	Units	Minimum	Maximum	Mean	Std. dev.	Trans	Model
Y1	N–N_2O	μg	0.001	2.75	0.87	0.79	Square root	Linear
Y2	COD	% removal	2.634	74.15	30.17	19.01	None	Quadratic
Y3	N–NH_3	% removal	0.001	98.20	48.71	31.99	None	Quadratic
Y4	TVSS	mg	0.002	72.64	38.20	25.48	None	Quadratic

Solutions

	Factors			Responses				Desirability
Number	N–NH_3	COD	Time	N–N_2O	COD	N–NH_3	TVSS	
1	107.79	2.89	40.000	1.35	41.06	67.94	58.66	0.72

Best fit models

Responses	Coded factors	Model *F* value	*P* value Prob > *F*	Remarks	R^2	Adequate precision
N–N_2O	$(N–N_2O)^{0.5} = 0.83 + 0.33C$	13.00	0.002	Significant	0.42	10.38
COD	$COD = 35.17 + 0.75A - 14.61B + 10.13C + 3.66AB + 7.93AC - 3.5BC - 7.66A^2 + 4.43B^2 - 4.08C^2$	18.81	<0.0001	Significant	0.94	15.81
N–NH_3	$N–NH_3 = 57.17 - 29.83A + 13.18B + 11.6C + 3.69AB + 1.65AC + 5.74BC + 1.80A^2 - 8.24B^2–5.95C^2$	17.24	<0.0001	Significant	0.94	15.89
TVSS	$TVSS = 59.02 - 0.26A + 14.83B + 12.50C + 9.68AB + 5.86AC + 7.66BC–13.38A^2 - 9.73B^2 - 7.38C^2$	7.01	0.0027	Significant	0.86	9.33

Study type: response surface; design type: central composite; design model: quadratic: runs: 20.

All the responses fit to the quadratic model except N–N_2O which follows linear model. In case of COD, the model F value is observed to be 18.81, which describes the model to be significant. The Prob > F value ⩽ 0.0049 indicates the model term to be significant. Adequate precision were taken so that the measure of signal-to-noise ratio should be ⩾ 4. So in conclusion, all the statistical values are significant, which shows the validity of the model. Similar values are obtained for all other responses as well as sets. A number of solutions were available but the maximum desirability was chosen.

In the present study, four responses were considered such as N–N_2O, N–NH_3, COD and TVSS along with three factors like time, COD and N–NH_3. To evaluate the overall design, the effect of each factor on the particular response was considered using the contour and 3D plots. The surface and contour plots using the quadratic model were developed by keeping one variable constant at a time. The optimized contour and 3D plots for responses like N–NH_3, TVSS and COD are shown in Fig. 6(b)—(d). Fig. 6(b) shows the contour and 3D plot for two variables like COD and N–NH_3 keeping the other variable, i.e. time constant at 40 h. The N–NH_3% removal increased with the increase of COD concentration and at lower N–NH_3 concentrations. More than 80% N–NH_3 removal could be obtained when COD and N–NH_3 concentration

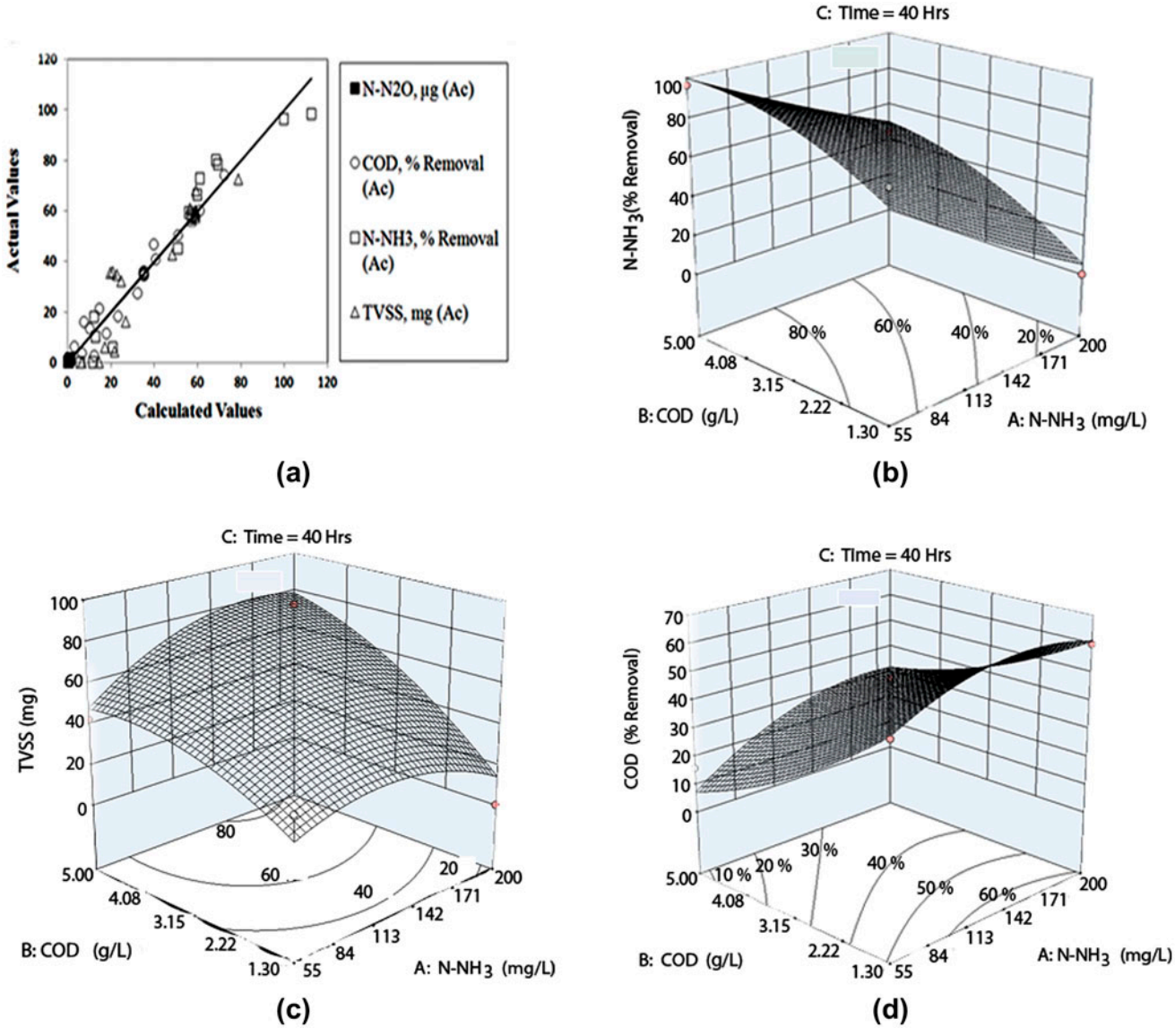

Fig. 6. Predicted vs. actual plot for responses like N–N_2O, COD, TVSS and N–NH_3 (a). Contour with 3D plots showing the optimization of four responses like N–NH_3, TVSS and COD, ((b)–(d)).

ranged between 2–5 g/L and 55–110 mg/L, respectively. Lower N–NH_3 removal efficiency is observed at higher N–NH_3 with lower COD concentrations. The reciprocal relations between COD and N–NH_3 can be explained through Eq. (2). In HBC route, the biomass uses both N–NH_3 and COD at a stoichiometric ratio of 1:1.18. Therefore, at lower N–NH_3 and higher COD concentrations, the removal efficiency of the former would be preferred. Fig. 6(c) shows the plot of two variables like COD and N–NH_3 keeping the other variable (time) constant at 40 h. 80% TVSS formation could be achieved in presence of COD and N–NH_3 concentrations ranging between 4.7–5 g/L and 145–190 mg/L, respectively. Fig. 6(d) shows the plot for two variables like COD and N–NH_3 keeping the other variable (time) constant at 40 h. The% of COD removal increased with the decrease of COD concentration and at higher N–NH_3 concentrations. About 60% of COD removal was obtained at COD and N–NH_3 concentration ranging between 1.3–1.6 g/L and 90–200 mg/L respectively. The optimized value for response N–N_2O is shown in the solution part of Table 5.

4. Conclusions

The COD and N–NH_3 removal rates varied between 0.5 to 1.9 mg/L/h (N–NH_3) for $(NH_4)_2SO_4$. The N–N_2O emission was observed to be very marginal. Detailed statistical analysis like ANOVA, PCA and MLRA were carried out. The ANOVA analyses suggest the significance of incubation period, i.e. time as well as nutrient concentrations. The PCA showed that the variables can be accommodated in two components explaining a total of 65.97% variance. MLRA were carried out between dependent variables like TVSS and N–NH_3 concentration respectively. Optimization studies were carried out to minimize N_2O emission, maximize the TVSS production along with simultaneous maximization for the removal efficiency of COD and N–NH_3. The optimum values of the process time, initial N–NH_3, and COD concentration in the aqueous solution were found to be 40 h, 107.79 mg/L, and 2.89 g/L, respectively. During the optimal values of the process parameters, a maximum N–NH_3 removal of 67.94% and COD removal of 41.06% were obtained. Also the N–N_2O formation was minimized to 2.96 μg along with 58.66 mg of TVSS production. The total desirability factor obtained was 0.72.

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References

[1] S. Ray, A. Mohanty, S.S. Mohanty, S. Mishra, G. Roy Chaudhury, Removal of nitrate and COD from wastewater using denitrification process: Kinetic, optimization, and statistical studies, Clean Technol. Environ. Policy (2013), doi: 10.1007/s10098-013-0621-7.
[2] M.J. Kampschreur, H. Temmink, R. Kleerebezem, M.S.M. Jetten, M.C.M. Van Loosdrecht, Nitrous oxide emission during wastewater treatment, Water Res. 43 (2009) 4093–4103.
[3] D.W. Koren, W.D. Gould, P. Bedard, Biological removal of ammonia and nitrate from simulated mine and mine effluents, Hydrometallurgy 56 (2000) 127–144.
[4] J. Foley, D. De Hass, Z. Yuan, P. Lant, Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants, Water Res. 44 (2010) 831–844.
[5] Z. Dai, C.C. Trettin, C. Li, H. Li, G. Sun, D.M. Amatya, Effect of assessment scale on spatial and temporal variations in CH_4, CO_2 and N_2O fluxes in a forested wetland, Water Soil Air Pollut. 223 (2012) 253–265.
[6] G. Tallec, J. Garnier, G. Billen, M. Gousailles, Nitrous oxide emissions from denitrifying activated sludge of urban waste water treatment plants, under anoxia and low oxygenation, Bioresour. Technol. 99(7) (2008) 2200–2209.
[7] Y. Toma, F. Takakai, U. Darung, K. Kuramochi, S.H. Limin, S. Dohong, R. Hatano, Nitrous oxide emission derived from soil organic matter decomposition from tropical agricultural peat soil in central Kalimantan, Indonesia, Soil Sci. Plant Nutr. 57(3) (2011) 436–451.
[8] W.H. Yang, Y.A. The, W. Silver, A test of a field-based ^{15}N-nitrous oxide pool dilution technique to measure gross N_2O production in soil, Glob. Change Biol. 17 (2011) 3577–3588.
[9] A. Bergstermann, L. Cardenas, R. Bol, L. Gilliam, K. Goulding, A. Meijide, D. Scholefield, A. Vallejo, R. Well, Effect of antecedent soil moisture conditions on emissions and isotopologue distribution of N_2O during denitrification, Soil Biol. Biochem. 43 (2011) 240–250.
[10] N. Wrage, G.L. Velthof, M.L. Van Beusichem, O. Oenema, Role of nitrifier denitrification in the production of nitrous oxide, Soil Biol. Biochem. 33 (2001) 1723–1732.
[11] N.D. Berge, D.R. Reinhart, J. Dietz, T. Townsend, *In situ* ammonia removal in bioreactor landfill leachate, Waste Manage. 26 (2006) 334–343.
[12] J.M. Ebeling, M.B. Timmons, J.J. Bisogni, Engineering analysis of the stoichiometry of photo autotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems, Aquaculture 257 (2006) 346–358.
[13] Q. Chen, J. Ni, Heterotrophic nitrification-aerobic denitrification by novel isolated bacteria, J. Ind. Microbiol. Biotechnol. 38 (2011) 1305–1310.
[14] I. Schmidt, O. Sliekers, M. Schmid, E. Bock, J. Fuerst, J.G. Kuenen, M.S.M. Jetten, M. Strous, New concepts of microbial treatment processes for the nitrogen removal in waste water, FEMS Microbiol. Rev. 27 (2003) 481–492.

[15] S.A. Balagodatsky, M. Kesik, H. Papen, K. Butterbach Bahl, Production of NO and N_2O by heterotrophic nitrifier *Alcaligenes faecalis* parafaecalis under varying conditions of oxygen saturation, Geomicrobiol. J. 23 (2006) 165–176.
[16] M.A. Zahed, H.A. Aziz, M.H. Isa, L. Mohajeri, Response surface analysis to improve dispersed crude oil biodegradation, Clean-Soil, Air, Water. 40 (2012) 262–267.
[17] K.S. Rao, S. Anand, K. Rout, P. Venkateswarlu, Response surface optimization for removal of cadmium from aqueous solution by waste agricultural biosorbent *Psidium guvajava* L. Leaf powder, Clean-Soil, Air, Water. 40 (1) (2012) 80–86.
[18] S. Aber, M. Sheydaei, Removal of COD from industrial effluent containing indigo dye using adsorption method by activated carbon cloth: Optimization, kinetic, and isotherm studies, Clean-Soil, Air, Water. 40(1) (2012) 87–94.
[19] J. Andert, E. Wessen, G. Borjesson, S. Hallin, Temporal changes in abundance and composition of ammonia-oxidizing bacterial and archaeal communities in a drained peat soil in relation to N_2O emissions, J. Soils Sediments. 11 (2011) 1399–1407.
[20] M. Heincke, M. Kaupenjohann, Effects of soil solution on the dynamics of N_2O emissions: A review, Nutr. Cycl. Agroecosys. 55 (1999) 133–157.
[21] APHA, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association and Water Environment Federation, Washington, DC, 2005.
[22] D. Pradhan, D. Mishra, D.J. Kim, J.G. Ahn, G. Roy Chaudhury, S.W. Lee, Bioleaching kinetics and multivariate analysis of spent petroleum catalyst dissolution using two acidophiles, J. Hazard. Mater. 175 (2010) 267–267.
[23] G.E.P. Box, J.S. Hunter, W.G. Hunter, An Introduction to Design Data Analysis and Model Building, John Wiley, New York, NY, 1978.
[24] E.W.J. Van Niel, P.A.M. Arts, B.J. Wesselink, L.A. Robertson, J.G. Kuenen, Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures, FEMS Microbiol. Ecol. 102 (1993) 109–118.
[25] O. Mathieu, J. Leveque, C. Henault, M.J. Milloux, F. Bizouard, F. Andreux, Emission and spatial variability of N_2O, N_2 and nitrous oxide mole fraction at the field scale, revealed with ^{15}N isotopic techniques, Soil Biol. Biochem. 38 (2006) 941–951.
[26] D. Mishra, J.G. Ahn, D.J. Kim, G. Roy Chaudhury, D.E. Ralph, Dissolution kinetics of spent petroleum catalyst using sulfur oxidizing acidophilic microorganisms, J. Hazard. Mater. 167 (2009) 1231–1236.
[27] A. Converti, A.D. Borghi, S. Arni, F. Molinari, Linearized kinetic models for the simulation of the mesophilic anaerobic digestion of pre-hydrolyzed woody wastes, Chem. Eng. Technol. 22(5) (1999) 429–437.
[28] J.F. Hair, R.E. Anderson, R.L. Tatham, W.C. Black, Multivariate Data Analysis, Prentice-Hall International, Upper Saddle River, NJ, pp. 87–138, 1998.
[29] A. Bell, T. Sejnowski, The independent components of natural scenes are edge filters, Vision Res. 37 (1997) 3327–3338.
[30] D.C. Lay, Linear Algebra and Its Applications, Addison-Wesley, New York, NY, pp. 441–486, 2000.
[31] S. Kong, B. Lu, Y. Ji, X. Zhao, Z. Bai, Y. Xu, Y. Liu, H. Jiang, Risk assessment of heavy metals in road and soil dusts within PM 2.5, PM 10, and PM100 fractions in Dongying city, Shandong province, China, J. Environ. Monit. 14 (2012) 791–803.