Orthogonal Test Design Based Analysis of Factors Controlling Simultaneous Anaerobic Sulfide and Nitrate Removal Process

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Summary: The present study deals with comprehensive analysis of the main factors like the effects of pH, influent substrate concentration and hydraulic retention time (HRT) driving simultaneous anaerobic sulfide and nitrate removal process. The analysis involved a multi-factorial orthogonal experiment. The results of range and variance analyses showed that decreasing influent substrate concentration might improve the effluent quality. Consequent to controlling the reaction pH at 7.0±0.1, the effluent quality was the best keeping influent sulfide concentration of 220 mg·L^{-1} and HRT of 10 h. Influent substrate concentration and HRT had significant bearing on the substrate removal rate, while pH had no significant effect. Decreasing HRT was a better option to increase substrate removal rate. Controlling the reaction pH around 7.0±0.1, feeding influent sulfide concentration of 520 mg·L^{-1} at HRT of 4h resulted in the optimum substrate removal rate.

Keywords: anaerobic sulfide and nitrate removal; pH; influent substrate concentration; HRT; orthogonal test design

Introduction

A number of industries generate sulfide-containing waste streams, such as petrochemical plants, tanneries, viscose rayon factories etc., [1]. Various toxicological effects of sulfide on human health have been described elsewhere; hence, it should be removed from wastewater before its discharge into the environment [2]. Various physicochemical and biological processes may be applied for the treatment of sulfide [3]. Physicochemical technologies are costly and generally require high energy inputs. In contrast, biological processes are cost effective that operate at prevailing environmental conditions without any requirement for expensive chemicals and catalysts [4].

Biological sulfide oxidation under aerobic conditions has attracted much attention and had been extensively studied [5]; however, oxygen injection requires energy which makes the operation costly. As an alternative to oxygen, nitrate or even nitrite can be used to control sulfide generation when treating the sulfide-containing wastewaters [6]. It was demonstrated that some bacterial species like *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* can oxidize sulfide to elemental sulfur simultaneously reducing nitrate or nitrite to dinitrogen. For such reasons, the simultaneous anaerobic sulfide and nitrate removal process has been recently developed. Our research group has done some work on the process performance and optimization. The effects of pH, influent substrate concentration and hydraulic retention time (HRT) on the process performance were studied by Cai et al. [7]. Some other researchers also paid attention to that process biochemistry [8-12].

However, it is common observation that wastewater treatment processes are often subjected to variations in one or more operational parameters, which would affect the overall process performance. All the factors definitely influence process performance, but the degrees of the influence would be variable. Deteriorated performance can be effectively corrected by searching the decisive factor which has the maximum bearing on the performance. During actual engineering practice, it is very meaningful to judge the critical factor. Recently, the research has mostly focused on the effect of single factor regarding the performance of simultaneous anaerobic sulfide and nitrate removal process. However, it is difficult to distinguish the degrees of influences of different factors through single-factor method. Literature review suggested that few reports existed highlighting the role of interaction among various factors influencing the process. The objective of the current study was to elucidate the effects of pH, influent substrate concentration and HRT on process performance through the orthogonal experiments. Moreover, interactions among various factors were also investigated by range and variance analyses.

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Results and Discussion

Effects on Effluent Quality

In order to improve the effluent quality, there were three options, i.e. keeping reaction pH constant nearly 7.0±0.1, decreasing influent substrate concentration and increase of HRT (Table-1). It is easy to get conclusion from the experiment results. But it is hard to pick out the best option. Range analysis is a good choice to judge the critical factor.

Table-1 showed the results of orthogonal test for effluent quality; further range analysis was performed based on the obtained results. In Table-1, K1 was used to indicate the sum of level 1 in each column, and K2 was used to indicate the sum of level 2 in the same column. k1 and k2 were the average values of K1 and K2, respectively. The range of each column was the absolute subtraction of k1 and k2, which was named as R. The range revealed the effect of corresponding factor on detecting index. The bigger the range was, the greater the effect of that factor on detecting index was [13].

The degree of effects would be different when different options were chosen. Considering different detecting indices, the degree order was also variable. When the effluent sulfide concentration was regarded as detecting index, the order of effects for three factors was as following: influent substrate concentration > pH > HRT. Considering the effluent nitrate concentration as detecting index, the order was as under: influent substrate concentration > HRT > pH. For eight treatments, the optimum conditions were obtained as A2B2C1. When the reaction pH was controlled around 7.0±0.1, with influent sulfide concentration of 220 mg $S{\cdot}L^{-1}$ and HRT of 10h, the effluent substrate concentration was at its lowest value.

It was suggested that decreasing influent substrate concentration was the best option to improve effluent quality because it had greater influence on it. It is also meaningful for process performance to recover from a state of deterioration. When the reactor performance deteriorated due to extreme disturbances, the concentrations of residual substrates and accumulated intermediate products would be rather high, which might inhibit microbial growth and metabolism. Consequently, the inhibited microbial growth and metabolism could even deteriorate the process performance leading to a vicious circle. Such "collapsed" process may be the worst outcome that could not be able to recover. When influent substrate concentrations were decreased, substrate loading rates on activated sludge would be effectively reduced. That could alleviate the performance deterioration. Consequently, the effluent quality would tend to be improved. The strategy of decreasing influent substrate concentration was of universal applicability to recover the process performance. When the process performance for anaerobic ammonia oxidation (ANAMMOX) deteriorated, Tang undertook that strategy to recover process performance, which was very successful [14].

The effect of pH on the effluent sulfide concentration was greater than that on effluent nitrate concentration, which was related to the special chemical properties of sulfide. Influent sulfide was not only an energy source but it also posed toxicity to microorganisms. It would denature proteins found in the cytoplasm but could inhibit ferredoxin and cytochrome c synthesis. It may also interfere with the various coenzymes A and M sulfide linkages [15]. The free sulfide was the most toxic form, which was closely related to pH [16]. Hence, pH was an important factor controlling the effluent sulfide concentration.

Table-1: Range analysis of orthogonal experiment for effluent quality.

Number		pH	Concentration	pH ×Concentration	HRT	pH ×HRT	Concentration ×HRT	Effluent sulfide concentration /($mg{\cdot}L^{-1}$)	Effluent nitrate concentration /($mg{\cdot}L^{-1}$)
		A	B	A×B	C	A×C	B×C		
1		1	1	1	1	1	1	0.60	1.76
2		1	1	1	2	2	2	2.56	8.92
3		1	2	2	1	1	2	0.89	3.8
4		1	2	2	2	2	1	0.37	1.24
5		2	1	2	1	2	1	1.01	1.56
6		2	1	2	2	1	2	0.51	7.25
7		2	2	1	1	2	2	0.51	0.39
8		2	2	1	2	1	1	0.32	1.02
Effluent sulfide concentration	K1	4.42	4.68	3.99	3.01	2.32	2.30		
	K2	2.35	2.09	2.78	3.76	4.45	4.47		
	k1	2.21	2.34	2.00	1.51	1.16	1.15		
	k2	1.18	1.05	1.39	1.88	2.23	2.24		
	R	1.04	1.30	0.61	0.38	1.07	1.09		
Effluent nitrate concentration	K1	15.72	19.49	12.09	7.51	13.83	5.58		
	K2	10.22	6.45	13.85	18.43	12.11	20.36		
	k1	7.86	9.75	6.05	3.76	6.92	2.79		
	k2	5.11	3.23	6.93	9.22	6.06	10.18		
	R	2.75	6.52	0.88	5.46	0.86	7.39		

Range analysis was a method to directly analyze orthogonal test results. Variance analysis was another method which could further analyze the results. Variance analyses of orthogonal experiment on effluent sulfide and nitrate concentrations were performed using SPSS 16.0 software (Table-2). Besides the effects of three factors, it also determined the effects of the interaction among factors. The degree of the interaction effects on the effluent sulfide concentration and the effluent nitrate concentration also varied. When the effluent sulfide concentration was regarded as detecting index, the order of effects among three interactions was as following: influent substrate concentration×HRT > pH×HRT > pH×influent substrate concentration (Table-2). When the effluent nitrate concentration was taken as detecting index, following was the order: influent substrate concentration×HRT > pH×influent substrate concentration > pH×HRT (Table-2).

It was revealed that the influent substrate concentration-HRT interaction was the prime factor among three influencing interactions. Usually, the optimum pH value for microorganisms that remove sulfide and nitrate simultaneously was nearly neutral. However, there were some variations among the reported values by different researchers. Krishnakumar et al. studied anoxic H_2S oxidation under denitrifying conditions by an isolated *Thiobacillus denitrificans* [17]. They found the optimum pH value around 7.5.When pH was higher than 8.0, the removal rate markedly decreased. Yang et al. investigated anoxic sulfide oxidation and nitrate reduction in wastewater under sewer conditions [18]. They found that typical anoxic sulfide oxidation rates were 0.48 g $S \cdot m^{-3} \cdot h^{-1}$ at pH 7.0 and 0.62 g $S \cdot m^{-3} \cdot h^{-1}$ at pH 8.5. However, the simultaneous anaerobic sulfide and nitrate removal process would be unstable, if pH exceeded 9.0 [19]. The present results showed that regardless of pH controlling method, the pH value in the reactor was always less than 8.0, which did not reach a level that could considerably affect the process performance. It might be the reason that the interactions related to pH had less effect on effluent quality.

Effects on Process Capacity

In order to expand process capacity, there were three options, i.e. keeping reaction pH around 7.0±0.1, increasing influent substrate concentration or decreasing HRT (Table-3). It was very important to estimate the decisive factor, which was helpful to improve the capability for substrate removal. The results of orthogonal test for process capacity were shown in Table-3, and range analysis was performed subsequently.

The degrees of effects on sulfide and nitrate substrate removal rates were variable. When the sulfide removal rate was regarded as detecting index, the order of effects among three factors was as follows: influent substrate concentration > HRT > pH. Considering the nitrate removal rate as detecting index, the order was as under: HRT > influent substrate concentration > pH. For eight treatments, the optimum conditions were obtained as A2B1C2. The sulfide removal rate was the fastest when the reaction pH was controlled around 7.0±0.1, the influent sulfide concentration was 520 mg $S \cdot L^{-1}$, with HRT of 4h.

Table-2: Variance analysis of orthogonal experiment for effluent quality.

Factor	Effluent sulfide concentration					Effluent nitrate concentration				
	S	df	MS	F	Sig.	S	df	MS	F	Sig.
Corrected Model	2.783[a]	6	0.464	0.477	0.802	68.005[b]	6	11.334	4.176	0.358
Intercept	5.729	1	5.729	5.888	0.249	84.110	1	84.110	30.986	0.113
A	0.536	1	0.536	0.550	0.594	3.781	1	3.781	1.393	0.447
B	0.839	1	0.839	0.862	0.524	21.255	1	21.255	7.830	0.218
A×B	0.183	1	0.183	0.188	0.739	0.387	1	0.387	0.143	0.770
C	0.070	1	0.070	0.072	0.833	14.906	1	14.906	5.491	0.257
A×C	0.567	1	0.567	0.583	0.585	0.370	1	0.370	0.136	0.775
B×C	0.589	1	0.589	0.605	0.579	27.306	1	27.306	10.060	0.194
Error	0.973	1	0.973			2.714	1	2.714		
Total	9.485	8				154.830	8			
Corrected Total	3.756	7				70.720	7			

[a] R Squared =0.741 (Adjusted R Squared = 0.813)
[b] R Squared = 0.962 (Adjusted R Squared = 0.731)

Table-3: Range analysis of orthogonal experiment for process capacity.

No.		pH	Concentration	pH ×Concentration	HRT	pH ×HRT	Concentration ×HRT	Sulfide removal rate/ ($kg\cdot m^{-3}\cdot d^{-1}$)	Nitrate removal rate/ ($kg\cdot m^{-3}\cdot d^{-1}$)
		A	B	A×B	C	A×C	B×C		
1		1	1	1	1	1	1	2.53	0.46
2		1	1	1	2	2	2	6.2	1.04
3		1	2	2	1	1	2	1.06	0.22
4		1	2	2	2	2	1	2.29	0.58
5		2	1	2	1	2	1	2.53	0.52
6		2	1	2	2	1	2	6.23	1.15
7		2	2	1	1	2	2	1.07	0.23
8		2	2	1	2	1	1	2.29	0.59
Sulfide removal rate	K1	12.08	17.49	12.09	7.19	12.11	9.64		
	K2	12.12	6.71	12.11	17.01	12.09	14.56		
	k1	6.04	8.75	6.05	3.60	6.06	4.82		
	k2	6.06	3.36	6.06	8.51	6.05	7.28		
	R	0.02	5.39	0.01	4.91	0.01	2.46		
Nitrate removal rate	K1	2.30	3.17	2.32	1.43	2.42	2.15		
	K2	2.49	1.62	2.47	3.36	2.37	2.64		
	k1	1.15	1.59	1.16	0.72	1.21	1.08		
	k2	1.25	0.81	1.24	1.68	1.19	1.32		
	R	0.10	0.78	0.08	0.97	0.02	0.25		

Variance analyses of orthogonal experiment for sulfide and nitrate removal rates were shown (Table 4). Variance analysis indicated that the effects of influent substrate concentration and HRT on process capacity were significant ($P < 0.05$). Among various influencing factors, HRT was more significant than influent substrate concentration. Considering substrates toxicity, it was suggested that decreasing HRT was the best option to get greater process capacity. Equation 1 indicated that substrate removal rate was directly related to the influent substrate concentration and HRT [20]. The results of variance analysis were in accordance with the theoretical prediction.

$$L = \frac{(C \times \eta)Q}{V} = \frac{C \times \eta}{\theta} \qquad \text{(Eq 1)}$$

L was the substrate removal loading rate; C was the influent substrate concentration; η was the substrate removal percentage; Q was the influent flow; V was the reactor volume; θ was HRT.

During the practical operation, decreasing HRT strategy attained the faster substrate removal loading rate [18]. When HRT was kept constant at 10h, the substrate loading rate enhanced through increasing the influent substrate concentration and the maximum sulfide-sulfur and nitrate-nitrogen removal rates were 4.57 $kg\cdot m^{-3}\cdot d^{-1}$ and 0.59 $kg\cdot m^{-3}\cdot d^{-1}$, respectively. While, the influent sulfide–sulfur and nitrate–nitrogen concentrations were 520 $mg\cdot L^{-1}$ and 95.6 $mg\cdot L^{-1}$, respectively, the substrate loading rate enhanced through decreasing HRT, and the maximum sulfide-sulfur and nitrate nitrate-nitrogen removal rates were 16.1 $kg\cdot m^{-3}\cdot d^{-1}$ and 2.5 $kg\cdot m^{-3}\cdot d^{-1}$, respectively.

Based on variance analysis, the order of effects among three interactions was as under: influent substrate concentration×HRT > pH×influent substrate concentration > pH×HRT (Table-4). It showed that the influent substrate concentration-HRT interaction was the prime one among three interactions and that interaction was significant on sulfide removal rate. Similar results were obtained with the effect of influent substrate concentration-HRT interaction on effluent quality. When the process was subjected to concentration variations and HRT at the same time, great variations in the performance would have been observed. It was promising to achieve great process capacity by increasing influent substrate concentration or decreasing HRT. But it was also bad to improve the effluent quality. These options were “double-edged sword”. On the basis of meeting effluent quality, the concentration was increased as high as possible, and HRT was decreased as low as possible.

Table-4 Variance analysis of orthogonal experiment for process capacity.

Factor	Sulfide removal rate					Nitrate removal rate				
	S	df	MS	F	Sig.	S	df	MS	F	Sig.
Corrected Model	29.606[a]	6	4.934	2.467E4	0.005	0.804[b]	6	0.134	428.573	0.037
Intercept	73.205	1	73.205	3.660E5	0.001	2.868	1	2.868	9.178E3	0.007
A	0.000	1	0.000	1.000	0.500	0.005	1	0.005	14.440	0.164
B	14.526	1	14.526	7.263E4	0.002	0.300	1	0.300	961.000	0.021
A×B	5.000E-5	1	5.000E-5	.250	0.705	0.003	1	0.003	9.000	0.205
C	12.054	1	12.054	6.027E4	0.003	0.466	1	0.466	1.490E3	0.016
A×C	5.000E-5	1	5.000E-5	0.250	0.705	0.000	1	0.000	1.000	0.500
B×C	3.026	1	3.026	1.513E4	0.005	0.030	1	0.030	96.040	0.065
Error	0.000	1	0.000			0.000	1	0.000		
Total	102.811	8				3.672	8			
Corrected Total	29.606	7				0.804	7			

[a.] R Squared = 1.000 (Adjusted R Squared = 1.000)
[b.] R Squared = 1.000 (Adjusted R Squared = 0.997)

Experimental

Inoculum and Enrichment of Microbial Communities

Inoculum was collected from the anaerobic methanogenic reactor operated at Dengta wastewater treatment plant (WWTP) located in Hangzhou City of China. Its total solids (TS) and volatile suspended solids (VSS) were 95.03 $g \cdot L^{-1}$ and 68.68 $g \cdot L^{-1}$ respectively, with VSS/TS ratio of 0.72. The simultaneous anaerobic sulfide and nitrate removal reactor was operated under lithoautotrophic conditions where sulfide was used as electron donor and nitrate was employed as electron acceptor to accomplish denitrification. For initial one month, the reactor was fed with synthetic wastewater in order to acclimatize the bacteria to the new substrates and to enrich the sludge.

Synthetic Wastewater

The reactor was fed with synthetic influent containing $NaHCO_3$, $MgCl_2$, KH_2PO_4, (1 $g \cdot L^{-1}$ each). $(NH_4)_2SO_4$ (0.24 $g \cdot L^{-1}$) and trace element solution (1 $mL \cdot L^{-1}$). The composition of trace element solution used was according to Mahmood *et al.* [21]. The nitrate-nitrogen and sulfide-sulfur concentrations were added in the form of and potassium nitrate (KNO_3) and sodium sulfide ($Na_2S \cdot 9H_2O$), respectively, with their concentrations varying according to the type of experiment conducted.

Anaerobic Sulfide Oxidizing (ASO) Reactor

The simultaneous anaerobic sulfide and nitrate removal reactor was an upflow reactor with biomass retention and was operated in a continuous mode. The reactor was made of perspex with a working volume of 1.3 L. The synthetic influent was pumped through a peristaltic pump from a 10 L influent vessel to the reactor. A recycling pump was used to mix the influent (substrate) and sludge (biocatalyst) well and hence to decrease possible substrate inhibition. The ratio of recycling flow to the influent flow was set to about 2.5~3.0. The temperature of the reactor was controlled between 29ºC and 31ºC.

Experimental Design

In order to estimate the effects of pH (Factor A), the influent substrate concentration (short as concentration, Factor B) and HRT (Factor C) on the process performance, the effluent substrate concentration and substrate removal rate were regarded as detecting indices. In the paper, orthogonal tests with three factors (pH, influent substrate concentration and HRT) and two levels (level 1 and level 2) were applied (Table-5). Considering the interactions among various factors, $L_8(2^7)$ orthogonal test table was used (Table-6).

Table-5: Test factor level table.

Level	A	B	C
	pH	Influent substrate concentration	HRT
1	Keeping influent pH constant around 7.0±0.1	520 mg $S \cdot L^{-1}$	10 h
2	Keeping reaction pH constant around 7.0±0.1	220 mg $S \cdot L^{-1}$	4 h

Influent substrate concentration was represented as influent sulfide concentration, and S-Sulfide/N-Nitrate in the influent =5/2(mol/mol)

Table-6 Orthogonal test design $L_8(2^7)$

Number	Factor						
	A	B	A×B	C	A×C	B×C	error
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

After the adjusting the environment factors in each treatment, the reactor was operated until the effluent quality got stable. Then, the average effluent substrate concentration and substrate removal loading rate were analyzed as detecting indices.

Analytical Procedures

Influent and effluent nitrate-nitrogen, pH and sulfide were analyzed during the operation of ASO reactor. Nitrate-nitrogen (NO_3^--N) was analyzed through ultraviolet spectrophotometric screening method [22] on daily basis using spectrophotometer (Unico UV-2102 PC and 722S, China). The sulfide was determined by iodometric method and sulfate was measured through turbimetric method [22]. The pH was determined following standard method [22]. A three-point calibration of pH meter was performed on daily basis. Total solids (TS) concentration was determined according to gravimetric method at 103ºC [22] and volatile solids were analyzed through gravimetric method at 550 ºC [22].

Conclusions

1) The effects of pH, influent substrate concentration and HRT were not significant on effluent quality, and the effects of three interactions among various factors were not significant either. Decreasing influent substrate concentration could improve the effluent quality effectively.

2) The effects of influent substrate concentration and HRT were significant on the process capacity, but the effects of pH were non significant. The effect of the influent substrate concentration-HRT interaction was significant on the sulfide removal rate, while the other interactions were non significant. Decreasing HRT was a better option to get greater process capacity.

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