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Assessment of simultaneous autotrophic-heterotrophic denitrification with high removal of nitrogen, sulphur, and carbon: optimization through response surface methodology

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Abstract

BACKGROUND: Some liquid industrial wastes are rich in sulfur (S), nitrogen (N) and organic matter, causing eutrophication of lakes and rivers. These wastes are also highly toxic to aquatic organisms and pose serious health problems. Biological N removal is performed by denitrification. Heterotrophic denitrifiers oxidize organic matter to CO₂ (g) and reduce nitrate (NO₃⁻) to N₂ (g), whereas autotrophic denitrifiers oxidize reduced S compounds by reducing nitrate. Thus, a simultaneous denitrification process will allow for the removal of all these elements.

RESULTS: A response surface methodology (RSM) was used to analyze the effects of the ratios heterotrophic:autotrophic bacteria (H/A, 0.2-1.9), the molar Ac⁻(Acetate)/NO₃⁻ (0.2-1.05) and tiosulphate (S₂O₃²⁻)/NO₃⁻ (0.15-1.1) on the denitrification process. The highest concentrations of sulfate were obtained with H/A= 0.2; Ac⁻/NO₃⁻: 1.05 and S₂O₃²⁻/NO₃⁻: 0.91.

CONCLUSION: High NO₃⁻ (average 95.0±3.3%) removals were obtained in all experiments, the optimal values predicted by the RSM were obtained at H/A: 0.54, Ac⁻/NO₃⁻: 1.05 and S₂O₃²⁻/NO₃⁻: 0.41. When analyzing the removal of acetate, the recommended optimal operating values were: Ac⁻/NO₃⁻: 1.05; H/A: 0.37 and S₂O₃²⁻/NO₃⁻: 0.8-0.9.

Keywords: Autotrophic denitrification; heterotrophic denitrification; response surface methodology (RSM); simultaneous sulfur and carbon removal.

Introduction

The contamination of groundwater and fluvial waters with nitrogenous compounds is a serious environmental problem worldwide. Ammonium not only leads to eutrophication and severe ecological damage to lakes and rivers, but also is highly toxic to aquatic organisms and generates bad odors.^[1] Nitrate causes serious health problems such as methemoglobinemia and carcinogens.^[2] In addition, several wastewaters contain organic matter and some sulphur (S) compounds. The latter are highly toxic, corrosive and possess a high chemical oxygen demand.^[1]

In order to remove these contaminants, biological treatments are preferred over physical-chemical methods.^[2] Ammonia can be oxidized biologically to nitrate under aerobic conditions and then reduced to molecular nitrogen by the biological denitrification process, which can be performed under heterotrophic and autotrophic conditions in anoxic environments. The stoichiometric reactions between acetate/nitrate ($\text{Ac}^-/\text{NO}_3^-$) and thiosulphate/nitrate ($\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$) are shown in equations (1) and (2).^[3]

Heterotrophic denitrification can be a highly efficient process.^[4] However, such processes do not remove sulfur compounds. In this sense, autotrophic denitrification is a good alternative, as it generates less sludge and eliminates the need to add an external carbon source, thus reducing operating costs.^[2] However, this latter process is affected by the presence of organic matter, which is generally present in the wastewater,

reducing sulfate production.^[5,6] In addition, although some methods have been used to remove nutrient with lower or no C source addition by heterotrophic denitrification, also removing phosphate,⁷ they operated in two reactors and using sulfur instead phosphate. Given this background, simultaneous autotrophic-heterotrophic denitrification is a novel alternative for the removal of sulphur, carbon and nitrogen; in this regard, several studies have demonstrated the importance of controlling the ratios of carbon/nitrogen (C/N) and sulphur/nitrogen (S/N) in this process.^[3, 8-10]

An et al.^[8] and Cardoso et al.^[3] demonstrated that when sulfate is supplemented under the stoichiometric ratio (high S/N ratios), incomplete oxidation of sulphide to elemental sulphur is favored. However, Campos et al.^[11] demonstrated that when thiosulphate is used as an electron donor, complete oxidation to sulfate occurs even at high S/N ratios. In addition, a C/N ratio above the stoichiometric favors heterotrophic bacteria, generating a low sulfur removal.^[9,10]

In relation to growth rates, denitrifying autotrophic bacteria have a maximum growth rate of 0.11-0.26 h⁻¹.^[12,13] The results for denitrifying heterotrophic bacteria are more variable, with values ranging from 0.062 to 0.108 h⁻¹^[14] in simultaneous autotrophic-heterotrophic cultures.^[13]

In order to identify the parameters which optimize the removal of the different contaminants, response surface methodology (RSM) is a good alternative and has been successfully applied to bioprocesses, for instance, optimizing the bacterial production of oligodextran and cellulose in bioreactors.^[15-17] More recently, factors affecting the performance of *Pseudomonas* sp. H117, isolated from the Tang Yu reservoir (China), were evaluated comprehensively and the autotrophic and heterotrophic denitrification differences determined based on the RSM analysis.^[18] Species of denitrifying bacteria

are capable of reducing nitrate and oxidizing Fe^{2+} and the optimal temperature, pH and C/N ratio for denitrification coupled with Fe^{2+} oxidation in groundwater also were investigated using the RSM. ^[19] In the same way, central composite design (CCD) and RSM were applied to optimize the C/N and current density in a heterotrophic/biofilm-electrode autotrophic denitrification reactor. ^[20]

The objective of the present work was to obtain a response surface model that can adequately describe the simultaneous autotrophic - heterotrophic denitrification process and allow optimization of the removal of nitrate, thiosulphate and acetate.

Materials and methods

Enrichment cultures

Two separate enrichment Continuous Stirred Tank Reactors (CSTRs) with a volume of 1.5 L were used in the batch feed mode. The reactors were agitated at 150 rpm, and kept at a temperature of 30 ± 2 °C for 120 days. The reactors were fed twice a week with 1 L of basal mineral medium (pH 7.0) composed of (g/L): Na_2HPO_4 (1.5), KH_2PO_4 (0.3), NH_4Cl (0.1), NaNO_3 (1.2); and trace elements of 1 mL/L. The trace elements were composed of $\text{Na}_2\text{MoO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (30), $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.0), CaCO_3 (2.0), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.5), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.25), HCl (37%) (50), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.25), H_3BO_3 (0.5). ^[21] The autotrophic medium was supplemented with (g/L): NaHCO_3 (1.5) and $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (5.0), while the heterotroph one was supplemented with (g/L): CH_3COOK (1.52). The autotrophic reactor was inoculated with 500 mL of anaerobic sludge (10 ± 0.6 g of volatile suspended solids (VSS)/L) from an anaerobic pond which treats pig slurry, and the heterotroph one was inoculated with 240 mL of an anaerobic sludge (31.1 ± 5 g VSS/L) from a digester which treats tobacco

residues (Chile). The head space of both reactors was purged with helium gas (5 min.). The two reactors were monitored once a week for Chemical Oxygen Demand (COD) (heterotrophic), nitrate, sulfate (autotrophic) and pH throughout the study.

Experimental design

A CCD was chosen to model the response surface, which required 15 experimental runs.^[22, 23] Each experimental run consisted of batch trials. The level of each variable is illustrated in Table 1. All analytical measurements were analyzed according to Table 2.

A response surface model was adjusted to the three measured variables: nitrate removal (%), acetate removal (%), sulfate production (mg/L) using Minitab's Experimental Design software 17.

Batch tests

The experiments were performed in Erlenmeyer flasks (100 mL) supplemented with 40 mL of medium. The reactors contained the above-mentioned basal mineral medium, NaNO₃ (10 mM) and varying concentrations of thiosulphate (1.5, 6.25 and 11.1 mM) and acetate (2, 6.25 and 10.5 mM). The reactors were inoculated with a total concentration of 1 g VSS/L; the proportion of autotrophic-heterotrophic bacteria for the inoculum was adjusted according to the quantification of the cultures obtained from the enrichment reactors, taking into account the levels of the variables described in the experimental design. The head space of the reactors was purged with Argon (2 min.) and the reactors were sealed with rubber and Teflon caps. Controls without inoculum were run in parallel to monitor the abiotic degradation. All batch assays were performed

in duplicate, and the center points in triplicate. All analytical measurements were performed in duplicate and analyzed according Table 2. The batch reactors were incubated in a thermostatic bath (30 °C and 150 rpm). The reactors were removed at 30 h of agitation and nitrate, thiosulfate, acetate, as well as sulfate production were measured, and the pH was monitored.

Results

The effect on the simultaneous denitrification of a low (0.2), medium (1.05) and high (1.9) ratio of Heterotrophic/Autotrophic bacteria was analyzed, along with a low (0.2), medium (0.63) and high (1.05) $\text{Ac}^-/\text{NO}_3^-$ ratio and a low (0.15), medium (0.63) and high (1.1) thiosulfate/nitrate ratio. The concentrations of nitrate, sulfate, acetate and thiosulfate were quantified at the initial time and at the end of the batch tests (30 hours). The time of the batch assays was determined from previous tests where samples were taken and the substrate and product concentrations were analyzed at regular time intervals for up to 80 hours. In addition, pH measurements, nitrate removal, sulfate production and COD were evaluated in the enrichment reactors throughout the study.

In each experimental run two control reactors (without inoculum) were used, and nitrate, sulfate and thiosulfate concentrations were determined. No nitrate and no thiosulfate degradation were observed in the controls in any of the trials ($P < 0.05$).

In all of the experiments, high NO_3^- removal rates ($95.0\% \pm 3.3\%$) were obtained (Figure 1a), which is indicative of an efficient denitrification process. The lowest percentages of nitrate removal were obtained in experiments 2, 5 and 7 corresponding to ratios of H/A (1.9, 1.9 and 0.2), of $\text{Ac}^-/\text{NO}_3^-$ (0.2, 1.05 and 0.2) and of $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ (0.15, 1.11 and 0.15).

On the other hand, when analyzing sulfate production (Figure 1b), it was observed that the lowest sulfate concentrations were achieved in experiments 2, 6 and 7 with an average value of 242 ± 6 mg/L, which were obtained in the lower limit of the ratio $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$, and were consistent with expectations. On the other hand, the highest concentration was observed in experiment 1 (1095 ± 38 mg/L), which had a low H/A ratio and a high $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ and $\text{Ac}^-/\text{NO}_3^-$ ratios. Experiment 3 agrees with these results (low H/A ratios and high $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratios), generating a high sulfate concentration, but a decrease in the ratio of acetate/nitrate to 0.2 brought about a decrease in sulfate concentration (14%).

In relation to the removal of acetate (Figure 1c), experiment 7 had the lowest removal ($40.7\% \pm 1.6\%$) with an H/A ratio of 0.2, $\text{Ac}^-/\text{NO}_3^-$ ratio of 0.2 and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ of 0.15. These results were consistent with those obtained for nitrate removal and sulfate production, indicating that a H/A bacteria ratio $< 1:1$ and poor nutrient availability, autotrophic-heterotrophic denitrification occurred inefficiently. In most of the other experiments, high acetate removals ($> 76\%$) were obtained, except for experiment 12 ($66.9\% \pm 0.4\%$), which presented stoichiometric ratios for acetate and thiosulfate and a low H/A ratio.

Using the data obtained in the 15 experimental runs, a response surface model was fitted using the Minitab software¹⁷, and the model was validated by verifying that it accurately described the observations and that the errors followed a normal and independent distribution with zero mean and constant but unknown σ^2 variance.^[23] This was done by analyzing the residuals (data not shown).

By analyzing the variance obtained for the response surface model of the nitrate removal, the "*P*-value" of the error obtained due to the non-fit of the model (0.002) was

lower than the alpha used (0.1). Then, the error due to lack of adjustment was justified by pure error. ^[24] The latter was given by the number of replicates and the experimental error, verifying that the model obtained was valid. The significant parameters and the final model are illustrated below:

Nitrate removal [%]

$$= 97.873 - 1.151 \frac{H}{A} + 1.798 \frac{CH_3COO^-}{NO_3^-} - 3.62 \frac{S_2O_3^{2-}}{NO_3^-} \cdot \frac{S_2O_3^{2-}}{NO_3^-} \\ - 1.78 \frac{H}{A} \cdot \frac{S_2O_3^{2-}}{NO_3^-} - 3.483 \frac{CH_3COO^-}{NO_3^-} \cdot \frac{S_2O_3^{2-}}{NO_3^-}$$

The main effects for nitrate removal (Figure 2), consisting of graphs of the mean marginal responses for the levels of the three factors analyzed, were assessed. ^[23] The three variables had a significant influence on nitrate removal ($P > 0.9$), and nonlinear influence for the variables H/A and $S_2O_3^{2-}/NO_3^-$ was observed, where the presence of a maximum was detected and a practically linear ratio of Ac^-/NO_3^- was observed with a positive slope.

The response surface graph for nitrate removal (Figure 3a) showed that the curvature of the surface indicates the quadratic character of the independent variable, and the presence of an optimal range for nitrate removal can be seen in the contour graph (Figure 3b). The optimal values predicted by the optimization tool of the software Minitab17 for nitrate removal were obtained at the ratios H/A of 0.54, Ac^-/NO_3^- of 1.05 and $S_2O_3^{2-}/NO_3^-$ of 0.41.

The same process then was performed to analyze the response surface model for the production of sulfate. The analysis of variance yielded a P -value of the error for lack of fit of the response surface model obtained for the sulfate production of 0.003 ($P < 0.1$);

this then supported the idea that the model was correct.^[23] The model with its relevant parameters is shown below:

Sulfate production [mg/L]

$$= 701.8 - 102.8 \frac{H}{A} + 190.5 \frac{S_2O_3^{2-}}{NO_3^-} - 108.8 \frac{H}{A} \cdot \frac{CH_3COO^-}{NO_3^-}$$

Next, it was determined whether the model adequately described the phenomenon and if it was possible to support the maximum or minimum found (in case it exists) from a theoretical point of view.^[23] Only two of the three independent variables studied had a significant influence on the sulfate concentration ($P > 0.9$), the H/A ratio or the $S_2O_3^{2-}/NO_3^-$ ratio. This is reflected in the graph of the main effects (Figure 4) where it can be seen that the variables had a nonlinear effect on the sulfate concentration, and presented a maximum for the H/A ratio and for the $S_2O_3^{2-}/NO_3^-$ ratio. Although a slight decrease due to the Ac^-/NO_3^- ratio was observed, it was not significant and can be attributed to an experimental error in the analytical technique for the measurement of sulfate.

In the response surface model for sulfate production (Figure 5a), a curvature was observed at the surface, indicating the quadratic character of the independent variable, and the presence of an optimal range for the production of sulfate was detected in the contour plot of the same factor (Figure 5b). The optimal values predicted by the software Minitab17 for sulfate production were obtained for an H/A ratio of 0.2, Ac^-/NO_3^- of 1.05 and $S_2O_3^{2-}/NO_3^-$ of 0.91.

In relation to acetate removal, the P -value for lack of fit of the model exceeded both the alpha (0.1) and the probability limit.^[23] In this sense, although the data were normal and it was possible to fit a model, the model obtained was not valid because its error was not justified by the pure error, which was given by the error of the experimental

replicates. Therefore, for the case of acetate, it was not possible to use a response surface model to find the optimal removal.

According to the results obtained, in order to favor the removal of thiosulfate, a greater number of autotrophic bacteria than the heterotrophic bacteria in the reactor was required; the optimal number found for sulfate production was at an H/A ratio of 0.2 (1:5). However, the optimal nitrate removal was found at an H/A ratio of 0.54 (1:1.85). At an H/A ratio of 0.2, a removal close to 98% was observed versus that of 100% for the optimum. Although the greatest difference in concentrations was observed for sulfate production, in order to favor both nitrate removal and thiosulfate removal it is recommended to operate the reactor at an H/A ratio of 0.37 (mean value). Another option could be to operate the reactor within a range of 0.2 to 0.54.

In the case of the acetate/nitrate ratio, an acetate stimulating effect was observed in the response surface model obtained for nitrate removal. Additionally, both optimization programs showed that the optimum is obtained at the highest analyzed level of the acetate-nitrate ratio, so that when operating the reactors, the optimum was chosen, $\text{Ac}^-/\text{NO}_3^-$ ratio = 1.05.

Finally, in the case of the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio, the maximum sulfate production was obtained at a ratio of 0.91. However, the maximum nitrate removal was obtained at a $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio of 0.41. In the case of nitrate removal, it was observed that at higher levels of thiosulfate, there was a decrease in nitrate removal, possibly due to an inhibitory effect of the thiosulfate on the heterotrophic bacteria. When referring to a decrease in nitrate removal, this means levels of 93% versus > 98% observed at the optimum. Taking into account all of these results, despite the fact that the optimal sulfate production was found at a $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio of 0.91, it is recommended that

slightly smaller ratios should be used to increase nitrate removal. In this sense, the reactor could be operated at a molar ratio of thiosulfate-nitrate in the range of 0.8-0.91.

Discussion

When analyzing the main effect of the H/A ratio on nitrate removal (Figure 3a), it was observed that at high level (1.9), where there was excess heterotrophic bacteria *versus* autotrophic bacteria, which are unable to use thiosulfate as electron donor, there was a nitrate removal of 95%. Thiosulfate had an inhibitory effect for *T. denitrificans* at a concentration of 17.86 mM,^[1, 12] and although all the media used had thiosulfate concentrations lower than this, it is possible that the remaining thiosulfate exerted an inhibitory effect on the denitrifying heterotrophs and, thus, on the denitrification process. This inhibitory effect hypothesis is consistent with the results obtained (Figure 1): high concentrations of thiosulfate (i.e. greater $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio analyzed) would inhibit the heterotrophic bacteria and generate a decrease in the efficiency of the denitrification process. The growth rate of heterotrophic microorganisms has been shown to decrease to a value of 0.047 h^{-1} in simultaneous autotrophic-heterotrophic cultures, where sulfide inhibition was observed.^[13] At the medium H/A ratio (1.05) and the low level (0.2), where there is an excess of autotrophic bacteria versus heterotrophic bacteria (5:1), nitrate removal levels close to 98% were observed. Therefore, the maximum nitrate removal was between these two levels.

In the case of the main effect of the $\text{Ac}^-/\text{NO}_3^-$ ratio on nitrate removal (Figure 3b), a linear trend with a positive slope was observed. This result would indicate that the acetate had a positive influence on nitrate removal in the simultaneous heterotrophic-autotrophic denitrification process. Although Beristain-Cardoso et al.^[9] and Chen et

al.^[10] reported heterotrophic bacteria were favored with a C/N ratio above stoichiometric, the results of the present research show a stimulatory effect of acetate. This is consistent with the results obtained by Cardoso et al.^[3] in pure autotrophic cultures, where it was observed that the lag phase was shortened and denitrification rates were increased with 0.5 mM acetate, which was explained by the fact that acetate can be used and assimilated as a carbon source by autotrophic microorganisms faster than inorganic carbon. Kuenen et al.^[24] reported that exogenous carbon sources can contribute to up to 30% of total cellular carbon in autotrophic denitrifiers such as *T. denitrificans*.

In the case of the main effect of the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio, it can be seen that lower levels of nitrate removal (95%-93%) were found at low and high levels (0.15 and 1.11), and that the maximum removal (98%) was achieved at a sub-stoichiometric ratio (0.625 equivalent to medium ratio). This can be explained by analyzing the studies carried out by An et al.^[8] where it was shown in batch tests that when sulfuric acid-nitrate ratios increased, the rate of nitrate removal increased and then decreased due to inhibition by hydrogen sulfide (prolonged lag phase). On the other hand, sulfate was the product of the process and a partial inhibition was reported at concentrations of 500 mg SO_4^{2-} -S/L and complete inhibition of activity at 6,400 mg SO_4^{2-} -S/L^[11,12,21] The inhibition by sulfate, as well as the influence of the S/N ratio on the denitrification process and the formation of the final products and their intermediates, could explain why there was a maximum value for nitrate removal for the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio.

The optimum removal obtained for nitrate was consistent with the results already described for the main effects. Thus, as in Figure 1, where it was observed that the higher concentrations of thiosulfate analyzed, as well as an excess of heterotrophic bacteria compared to the autotrophic bacteria in experiment number 5 (H/A ratio of 1.9

and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ of 1.11), were not the ideal conditions for simultaneous autotrophic-heterotrophic denitrification. In addition, the main factors indicate that in order to favor the removal of nitrate in synthetic wastewater, a greater amount of denitrifying autotrophic bacteria than heterotrophic bacteria was required, specifically at a ratio of 1.85:1. This supports the idea that high thiosulfate/nitrate ratios inhibit heterotrophic bacteria by reducing the denitrification process, and that acetate has a stimulatory effect on the process.

In the case of the main effect of the H/A ratio on sulfate, the maximum sulfate production was found (Figure 5a) for the lowest ratio analyzed (0.2). This can be explained by the fact that high H/A ratios imply a low amount of denitrifying autotrophic bacteria in the culture medium, resulting in a lower sulfate concentration. By contrast, low H/A ratios increase sulfate concentration because more autotrophic bacteria exist than heterotrophic bacteria.

Figure 5b shows how the maximum sulfate production was found between the mean level (0.625) and the high level (1.11) of the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio. On the one hand, as described in the theoretical framework, several authors have demonstrated the influence of the S/N ratio on the denitrification process, showing that low S/N ratios, when nitrate is in stoichiometric concentrations or in excess, favor the conversion of sulfide acid to sulfate. On the other, at high S/N ratios, nitrate acts as a limiting factor and favors the partial reduction of sulfide acid to elemental sulfur. However, Campos et al.^[11] observed that when thiosulfate was used as an electron donor, elemental sulfur formation was not observed, even at high S/N ratios (6.67 g/g). The latter can be explained by the inhibitory effect of hydrogen sulfide at high concentrations (high S/N ratios), which was demonstrated by a prolonged lag phase and a decrease in the rate of oxidation in the denitrifying autotrophic/heterotrophic culture with a concentration of 16.3 mM of H_2S .

[8]. In a previous study which analyzed the kinetics of autotrophic denitrifiers using sulfur, hydrogen sulfide and thiosulfate as an electron donor, it was observed that the rate of denitrification and the oxidation of sulfur compounds depended greatly on the compound used and was very fast for thiosulfate, followed by hydrogen sulfide and much slower for elemental sulfur. It was also found that hydrogen sulfide inhibited the oxidation of sulfur compounds by decreasing the rate by approximately 21-fold when the concentration of hydrogen sulfide was increased from 2.5 to 10 mM.^[3]

Due to fact that in the present study the electron-donor compound was thiosulfate, it made sense that the maximum sulfate production should take place over the stoichiometric value of the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio. However, the optimal sulfate production was not found precisely at the high level of the variable, which is explained by the inhibitory effect of the same sulfate that began to generate partial inhibition at concentrations of 500 mg SO_4^{2-} —S/L.^[11,12,21]

The optimum found for the production of sulfate was consistent with the results already described for the main effects. Moreover, it can be observed in Figure 1b that the lowest concentrations of sulfate (Experiment 2, 6, 7), with an average of 242 ± 6 mg/L, were obtained near the lower limit of the $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio. However, the highest concentration of sulfate was observed in experiment 1 (1095 ± 38 mg/L), which had a low H/A ratio, and high $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ and $\text{Ac}^-/\text{NO}_3^-$ ratios. Experiment 3 is consistent with these results (low H/A ratios and high $\text{Ac}^-/\text{NO}_3^-$ ratios), and generated a high sulfate concentration, but when the acetate/nitrate ratio decreased to 0.2, a decrease in the sulfate concentration was observed (14%).

It has been reported by several authors that manipulating the S/N ratio in a reactor may favor the production of sulfate from synthetic wastewater.^[3, 8-10] The results indicate

that a greater amount of denitrifying autotrophic bacteria than heterotrophic bacteria is required, specifically a 5:1 ratio, to optimize SO_4^{2-} production. They support the idea that acetate has a stimulatory effect on the process and that high $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratios increase SO_4^{2-} production.

Conclusions

High nitrate removal was observed for all experiments ($95.0 \pm 3.3\%$), indicating the ability of both groups of bacteria to coexist with a synthetic wastewater medium which is rich in thiosulfate, nitrate and acetate. When using the response surface methodology (RSM) to model the simultaneous autotrophic-heterotrophic denitrification process, it was found that the maximum nitrate removal was achieved at H/A ratio of 0.54, $\text{Ac}^-/\text{NO}_3^-$ ratio of 1.05 and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio of 0.41. When using the RSM to model the process of simultaneous autotrophic-heterotrophic denitrification, it was found that the maximum sulfate production was reached at H/A ratio of 0.2; $\text{Ac}^-/\text{NO}_3^-$ ratio of 1.05 and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ of 0.91. Therefore, the optimal operation values recommended were: $\text{Ac}^-/\text{NO}_3^-$ molar ratio of 1.05, denitrifying H/A ratio of 0.37 (average value between 0.54 and 0.2) and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ ratio of 0.8-0.9. Finally, by manipulating the acetate, thiosulfate and proportion of bacteria, it was possible to favor the elimination of sulfur, carbon and nitrogen within the reactors.

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Table 1. Factors and levels of the experimental variables used in the experimental design to investigate the influence of the ratios of $\text{Ac}^-/\text{NO}_3^-$, $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ and H/A on the denitrification process.

Factors	Unit	Codified Factor	Level -1	Level 0	Level +1
Heterotrophs/Autotrophs(H/A)	VSS	X_1	0.2	1.05	1.9
$\text{Ac}^-/\text{NO}_3^-$	mM	X_2	0.2	0.63	1.05
$\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$	mM	X_3	0.15	0.63	1.11

Factors

Experiment

number	H:A	$\text{Ac}-\text{NO}_3^-$	$\text{S}_2\text{O}_3^{2-}:\text{NO}_3^-$
1	0.2	1.05	1.11
2	1.9	0.2	0.15
3	0.2	0.2	1.11
4	1.9	0.2	1.11
5	1.9	1.05	1.11
6	1.9	1.05	0.15
7	0.2	0.2	0.15
8	0.2	1.05	0.15
9	1.05	0.625	0.63
10	1.05	0.2	0.63
11	1.05	1.05	0.63
12	0.2	0.625	0.63
13	1.05	0.625	1.11
14	1.9	0.625	0.63
15	1.05	0.625	0.15

Table 2. Control parameters of the process, analyses carried out and analytical methods used.

Parameter	Methodology	Reference
Autotrophic and heterotrophic microorganisms	VSS	APHA ^[25]
Acetate*	Ionic Chromatography	Van Straten et al. ^[26]
Nitrate/Nitrite**	Spectrophotometry	APHA ^[25]
Sulfate**	Spectrophotometry	APHA ^[25]
Tiosulfate**	Iodometry	Vogel ^[27]
pH	Potentiometry	-

* Membrane filtration (0.45 µm) previously centrifuged at 10,000 rpm for 5 min.

** Vacuum filtration by cellulose membrane (5 µm).

FIGURE CAPTIONS

Figure 1. **a)** Percentage of nitrate removal within the reactors at 30 h of batch tests. Error bars represent an IC ($\alpha = 5\%$, SD, $n = 4$). **b)** Sulphate concentrations recorded in the reactors at 30 hours of the batch tests in the fifteen experimental runs. Error bars represent IC ($\alpha = 5\%$, SD, $n = 4$). **c)** Percentage of acetate removal in the reactors at 30 h of the batch tests, in the fifteen experimental runs. Error bars represent IC ($\alpha = 5\%$, SD, $n = 4$).

Figure 2. Graph of the main effects for the removal of nitrate from the three variables analyzed: H/A, $\text{Ac}^-/\text{NO}_3^-$ and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ (Minitab17).

Figure 3.a) Contour plot for the removal of nitrate *versus* the ratio H/A and the ratio $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ fixing the ratio $\text{Ac}^-/\text{NO}_3^-$ to 0. **b)** Response surface graph of nitrate removal as a function of the ratio H/A and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$, fixing the ratio $\text{Ac}^-/\text{NO}_3^-$ to 0 (Minitab17).

Figure 4. Graph of the main effects of the production of sulphate from the three variables analyzed: H/A, $\text{Ac}^-/\text{NO}_3^-$ and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ (Minitab17).

Figure 5. a) Contour plot for the production of sulphate *versus* the ratio H/A and the ratio $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$ fixing the ratio $\text{Ac}^-/\text{NO}_3^-$ to 0. **b)** Response surface graph of the production of sulfate as a function of the ratio H/A and $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$, fixing the ratio $\text{Ac}^-/\text{NO}_3^-$ to 0 (Minitab17).

a

b

c

Figure 1.

Figure 2.

a)

b)

Figure 3.

Figure 4.

a)

b)

Figure 5.