Evaluation of nitrate and perchlorate reduction using sulfur-based autotrophic and mixotrophic denitrifying processes
Deniz Uçar, Emine Ubay Çokgör and Erkan Şahinkaya

ABSTRACT
The biological reduction of nitrate and perchlorate was comparatively evaluated in autotrophic and mixotrophic bioreactors using elemental sulfur and/or methanol as the energy source. The mixotrophic reactor was supplemented with methanol at CH₃OH/NO₃-N ratio of 1 or 1.4. The mixotrophic reactor completely reduced perchlorate in the feed up to 1,000 μg l⁻¹. The autotrophic reactor also showed high perchlorate reduction performance and decreased perchlorate from 1,000 μg l⁻¹ to around 33 μg l⁻¹. Complete reduction of 25 mg NO₃-N l⁻¹ was achieved in both reactors, corresponding to a maximum nitrate reduction rate of 300 mg NO₃-N l⁻¹d⁻¹ and 400 mg NO₃-N l⁻¹d⁻¹ in the autotrophic and mixotrophic processes, respectively. Autotrophic denitrification caused an increase of effluent sulfate concentration, which may exceed the drinking water guideline value of 250 mg l⁻¹. In the mixotrophic denitrification process, the effluent sulfate concentration was controlled by adjusting the C/N ratio in the influent. Mixotrophic denitrification was stimulated by 25 mg l⁻¹ methanol addition and 53% of influent nitrate was reduced by the heterotrophic process, which decreased the effluent sulfate concentration to half of the autotrophic counterpart. Therefore, the mixotrophic process may be preferred over the autotrophic process when effluent sulfate concentration is of concern and a higher perchlorate reduction efficiency is desired.

Key words | autotrophic denitrification, mixotrophic denitrification, nitrate reduction, perchlorate reduction

INTRODUCTION
Elemental sulfur or elemental sulfur/methanol based upflow denitrifying bioreactors have been shown to simultaneously reduce multiple oxidized contaminants (Akunna et al. 1993; Sahinkaya & Kilic 2014). Nitrate is the most commonly encountered oxidized contaminant in water, which mainly originates from agricultural run-off and wastewater discharges and is a concern in drinking water since it can cause blue-baby syndrome (Ziv-El & Rittmann 2009). Nitrate may be found in water together with some other oxyanions such as perchlorate, selenate, trichloroethene, bromate, arsenic, etc. (Chung et al. 2007a, b).

Perchlorate (ClO₄⁻) is the salt derived from perchloric acid and is known to inhibit thyroid function (Motzer 2001). It usually presents in water sources as a result of improper disposal of solid rocket fuels containing ammonium perchlorate. Biological reduction is the most commonly used process for the detoxification of these oxyanions, due to fast reaction rate and the elimination of expensive catalysts or chemical requirements (Srinivasan & Sorial 2009).

Removal of perchlorate from drinking water sources needs to be considered together with nitrate mainly because (1) it usually presents in water sources at the μg l⁻¹ level, at which it is difficult to support bacterial growth and (2) perchlorate is generally present in water together with nitrate (McCarty & Meyer 2005). Several bioreactor technologies...
have been shown to be highly effective for the removal of ClO\textsubscript{4}\textsuperscript{-} (Ju et al. 2008; Fox et al. 2014). However, all bioreactor concepts studied to date rely on the continuous addition of an electron-donating substrate. Biological reduction of nitrate and perchlorate can be achieved using organic (Wang et al. 2012) or inorganic electron sources (Ju et al. 2008). Organic electron donors are fast and effective; however the main disadvantage of the process is the difficulty of dosing the proper amount of organic carbon. Heterotrophic denitrification with methanol as electron donor is also shown in Equation (1), which shows 2.47 g methanol is required for complete reduction of 1 g NO\textsubscript{3} to N\textsubscript{2} gas (Sahinkaya & Dursun 2012).

\[
\text{NO}_3^- + 1.08\text{CH}_3\text{OH} + 0.24\text{H}_2\text{CO}_3
\rightarrow 0.056\text{C}_3\text{H}_7\text{NO}_2 + 0.47\text{N}_2 + 1.68\text{H}_2\text{O} + \text{HCO}_3\quad (1)
\]

Acetate is the most commonly used electron donor in biological reduction of perchlorate (Bardiya & Bae 2011). Wang et al. (2013) compared the efficiency of hydrogen and acetate as electron donors for perchlorate reduction. Equations (2) and (3) show the basic perchlorate reduction with hydrogen (Nerenberg & Rittmann 2004) and acetate (Nor et al. 2011), respectively, as electron sources.

\[
\text{ClO}_4^- + 4\text{H}_2 \rightarrow \text{Cl}^- + 4\text{H}_2\text{O} \quad (2)
\]

\[
\text{CH}_3\text{COOH} + \text{ClO}_4^- \rightarrow 2\text{CO}_2 + \text{Cl}^- + 2\text{H}_2\text{O} \quad (3)
\]

The ratio of chemical oxygen demand (COD)/perchlorate also affects the perchlorate reduction rate and the efficiency. The optimum COD/perchlorate ratio was reported as 1.65 g COD/g perchlorate and 1.45 g COD/g perchlorate in the studies of Wang et al. (2013) and Nor et al. (2011), respectively. The optimum ratio corresponds to the minimum ratio, which resulted in the complete depletion of both perchlorate and acetate. Ricardo et al. (2012) reported over 95% nitrate (from 60 to 2.8 ± 0.5 mg l\textsuperscript{-1}) and 93% perchlorate (from 100 to 7 ± 0.8 µg l\textsuperscript{-1}) reduction with mixed anoxic culture in the presence of ethanol (Ricardo et al. 2012). If carbon is added at a concentration higher than the stoichiometric requirement, residual organic matter can stimulate bacterial growth in water distribution systems and contribute to the formation of disinfection byproducts during chlorination (Ju et al. 2008). In the contrary case, nitrite may accumulate, which is more toxic compared to nitrate. To overcome these problems, autotrophic perchlorate reducing bioreactors in which inorganic electron donors such as H\textsubscript{2} (Ziv-El & Rittmann 2009), Fe(0) (Cao et al. 2005) and elemental sulfur (Ju et al. 2008) have also been used as electron donors. Cao et al. (2005) investigated the perchlorate reduction with iron nanoparticles. The reaction was temperature dependent and the perchlorate reduction rate at 75 °C was 1.52 mg perchlorate/(g nanoparticles.h). Although the reaction is favorable in terms of thermodynamics (activation energy was calculated as 79.02 ± 7.75 kJ mole\textsuperscript{-1}), as the authors noted, perchlorate reduction is limited by the slow kinetics (Cao et al. 2005). Ju et al. (2008) tested the performances of various inoculums taken from aerobic or anaerobic environments with various electron donors. The reduction rate was 0.18 mmol l\textsuperscript{-1} d\textsuperscript{-1} with S\textsuperscript{0} and aerobic process sludge for electron donor and inoculum respectively. Reduction rates of hydrogen and Fe\textsuperscript{0} with the same inoculum were ≥0.37 mmol l\textsuperscript{-1} d\textsuperscript{-1} on day 8 and 0.085 mmol l\textsuperscript{-1} d\textsuperscript{-1} on day 37, respectively (Ju et al. 2008).

The sulfur packed bed denitrifying bioreactor is an effective and economical process (Demirel et al. 2014; Sahinkaya et al. 2015). Granular S\textsuperscript{0} provides a slow release of electrons on demand, offering the advantages of eliminating dose adjustment, in a simple and reliable operation. The expected stoichiometry of the reaction is as follows (Sahinkaya & Dursun 2012):

\[
55\text{S}^0 + 50\text{NO}_3^- + 58\text{H}_2\text{O} + 20\text{CO}_2 + 4\text{NH}_4^+ 
\rightarrow 4\text{C}_3\text{H}_7\text{O}_2\text{N} + 55\text{SO}_4^{2-} + 25\text{N}_2 + 64\text{H}^+ \quad (4)
\]

There are few studies on sulfur-based mixotrophic denitrification processes for drinking water treatment. Liu et al. (2009) combined the heterotrophic and sulfur based autotrophic process for nitrate reduction. When C/N ratio was 2, 30 mg l\textsuperscript{-1} NO\textsubscript{3}–N was completely reduced without excess sulfate production (<130 mg l\textsuperscript{-1}) (Liu et al. 2009). Autotrophic, mixotrophic and heterotrophic denitrification performances were compared by Oh et al. (2001). While the denitrification rate for a sulfur based reactor was 1.4 kg m\textsuperscript{-3} d\textsuperscript{-1}, it increased to 1.92 and 2.7 kg m\textsuperscript{-3} d\textsuperscript{-1} with 132.8 and 571.4 mg l\textsuperscript{-1} methanol supplementation (Oh et al. 2001). Sahinkaya & Dursun (2012) reported that
acidity produced by an S⁰ based autotrophic reactor was neutralized by the alkalinity produced by the heterotrophic process and complete reduction of 75 mg NO₃⁻-N l⁻¹ was achieved under mixotrophic conditions. Studies regarding dual reduction of nitrate and other oxyanions are also present in the literature. Sahinkaya et al. (2013) investigated the simultaneous nitrate and chromate reduction in an S⁰/methanol based mixotrophic process. Complete reduction of 75 mg NO₃⁻-N l⁻¹ and 10 mg l⁻¹ Cr(VI) were achieved under varying C/N ratios (1.33–2) and a hydraulic retention time (HRT) of 3.7 (Sahinkaya et al. 2013).

Perchlorate concentrations in drinking water sources are relatively low, in micrograms per litre range and it is difficult to add the exact amount of a single organic electron donor to reduce milligram range nitrate and microgram range perchlorate. Mixotrophic reduction of these oxyanions may overcome the organic contamination risk and it also provides satisfying sulfate and alkalinity concentrations in the effluent. In this context, this study aims at comparing the simultaneous nitrate and perchlorate bioreduction performances of elemental sulfur based autotrophic and mixotrophic denitrification processes. According to the best of the author’s knowledge, this is the first study on simultaneous reduction of perchlorate and nitrate in a sulfur-based mixotrophic denitrification process.

MATERIALS AND METHODS

Column bioreactors

Two laboratory-scale glass columns with an empty bed volume of 400 ml were used as bioreactors (autotrophic and mixotrophic). The autotrophic reactor was filled with elemental sulfur only, whereas the mixotrophic reactor was filled with elemental sulfur (0.5–1 mm) and limestone (0.5–1 mm). Based on the results of Kilic et al. (2014), the limestone to elemental sulfur ratio was 1:2 (Kilic et al. 2014). The use of small sulfur and limestone particles was not to limit the denitrification rate as the dissolution of sulfur depends on surface area. To prevent the growth of phototrophic bacteria, the columns were covered with aluminum foil. The reactors were fed continuously in up-flow mode using adjustable peristaltic pumps at 28–30 °C in a temperature controlled room.

Inoculation and operation of the reactors

A 50 ml (volatile suspended solids (VSS) = 26000 ± 430 mg l⁻¹) denitrifying activated sludge obtained from the first anoxic tank of a 5-stage Bardenpho process located in Harran University Campus (Sanliurfa, Turkey) was used as inoculum for the autotrophic reactor. Around 50 ml (VSS = 16000 ± 240 mg l⁻¹) denitrifying sludge obtained from another mixotrophic reactor was used for the inoculation of the mixotrophic bioreactor. The reactors were operated in batch mode for the first 3 days after inoculation and then were operated continuously in up-flow mode. The freshly prepared feed solution was deoxygenated by passing through the N₂ gas for 20 min. The feed was then kept under anaerobic conditions in collapsible feed containers. The reactor was fed with tap water supplemented with 50 mg l⁻¹ K₂HPO₄ as a source of phosphorus, and KNO₃ to obtain 25 mg NO₃⁻-N l⁻¹. To supply alkalinity in the autotrophic reactor, 375 mg NaHCO₃ l⁻¹ was added to the feed. The mixotrophic reactor was supplemented with methanol (25 and 35 mg l⁻¹) to provide an external carbon source. Operational conditions of the reactors are presented in Table 1.

Both reactors were operated under varying operational (Table 1) conditions to evaluate their effects on denitrification and perchlorate reduction performances as well as sulfate production. The reactors were sampled at least three times a week for the measurement of NO₃⁻-N, NO₂⁻-N, ClO₄⁻, ClO₃⁻, ClO₂⁻, Cl⁻, SO₄²⁻, HS⁻, dissolved organic carbon (DOC), pH and alkalinity. The feed solution was also sampled regularly for the determination of NO₃⁻-N, ClO₄⁻, Cl⁻, NO₂⁻-N, SO₄²⁻, DOC, pH, and alkalinity.

Batch adsorption experiments

Batch adsorption experiments were conducted to identify NO₃⁻ and ClO₄⁻ removal mechanisms. In the experiments, 100 ml serum bottles were supplemented with 25 mg l⁻¹ NO₃⁻-N and 1,000 μg l⁻¹ ClO₄⁻. For the autotrophic denitrification experiments, the serum bottles were supplemented with 1.5 g elemental sulfur. Similarly, for the mixotrophic denitrification experiments, the serum bottles were supplemented with 1.5 g elemental sulfur, 1 g limestone and 100 mg l⁻¹ CH₃OH. All serum bottles were operated
in a temperature controlled room at 28–30°C and regularly sampled for the measurements of NO₃-N, and perchlorate.

**Analytical methods**

Chlorate, chlorite, chloride, nitrate, nitrite, and sulfate were analyzed by suppressed conductivity ion chromatography using a Shimadzu HIC-SP system fitted with a DIONEX Ion-Pac AS9-HC column (4 mm × 250 mm with a detection limit of 0.1 mg l⁻¹). Perchlorate was measured by a DIONEX 500 system (suppressed conductivity) with Ion-Pac AS20 column (Detection limit was 2 μg l⁻¹). Dissolved organic carbon was measured by a total organic carbon (TOC) analyzer (Shimadzu, Japan). Alkalinity and COD were measured according to *Standard Methods* (APHA 2005). Sulfide was measured spectrophotometrically at 480 nm using a Shimadzu UV-VIS spectrophotometer following the method described by Cord-ruwisch (1985). Sodium perchlorate monohydrate and potassium nitrate were purchased from Sigma (St Louis, MO). Samples were filtered through 0.45 μm pore sized cellulose acetate syringe filters before ion and sulfide measurements.

### RESULTS AND DISCUSSION

#### Perchlorate reduction

Both autotrophic and mixotrophic reactors simultaneously reduced nitrate and perchlorate. Perchlorate concentration in groundwater was reported up to 1,000 μg l⁻¹ (Ye et al. 2015), which was considered the maximum level in our study. In the autotrophic reactor, perchlorate was added to the influent on day 298 and its concentration increased gradually up to 1,000 μg l⁻¹ at a constant nitrate loading rate of around 300 mg NO₃⁻N l⁻¹ d⁻¹ (Table 1). In period 4, perchlorate concentration in the influent and effluent of the autotrophic reactor averaged 50 μg l⁻¹ and 15.61 ± 11.80 μg l⁻¹ respectively, corresponding to 69% removal (Figure 1). Increasing perchlorate concentrations resulted in better perchlorate reduction efficiency and increase in reduction rate due to acclimation of the population to higher perchlorate loading, which is also supported by other studies (Webster et al. 2009).

#### Table 1

<table>
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<th>Periods</th>
<th>Days</th>
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<th>CH₃OH (mg l⁻¹)</th>
<th>ClO₄⁻ (μg l⁻¹)</th>
<th>Alkalinity (mg CaCO₃ l⁻¹)</th>
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<th>ClO₄⁻ (μg l⁻¹)</th>
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Accordingly, when the influent perchlorate concentration in the autotrophic process was further increased to 200 μg l⁻¹ in period 6 (Table 1), effluent perchlorate concentration in autotrophic bioreactor averaged 12.05 ± 10.3 μg l⁻¹, corresponding to 94% removal efficiency, which was similar to that obtained in period 5. Similar effluent perchlorate removal efficiencies of 97% and 96% were detected in the following periods 7 and 8, with effluent concentrations of 8.90 ± 9.36 and 21.07 ± 33.98, respectively. In the last period, around 97% perchlorate removal was obtained with influent and effluent concentrations of 1,000 μg l⁻¹ and 33.23 ± 30.4 μg l⁻¹, respectively (Figure 1).

Better reduction efficiencies were observed in the methanol/elemental sulfur based mixotrophic reactor. On day 59, perchlorate was added to the influent with 50 μg l⁻¹ and effluent perchlorate concentrations were always below the detection limit (2 μg l⁻¹) during this period. In the following period (days 74–102), influent perchlorate concentration was increased to 100 μg l⁻¹ and this decreased the removal efficiency from 100 to 75%. However, perchlorate concentrations in the effluent decreased down to under detectable levels in day 80 and remained the same until the end of this period. Further increase in perchlorate concentration to 200 μg l⁻¹ led to a decrease in performance down to 69%. Similarly, in the autotrophic reactor, as the bacterial population acclimated to higher concentrations, removal efficiencies recovered on day 110. Although increasing perchlorate concentrations led to a decrease in perchlorate removal efficiency, the reactor recovered its complete reduction performance (Figure 1). In the last period, complete reduction of 1,000 μg l⁻¹ perchlorate was accomplished. Intermediate products (ClO₂ and ClO₃) of perchlorate reduction were not detected throughout the operation of the autotrophic and mixotrophic reactor. The presence of other electron acceptors may competitively inhibit the system performance. Perchlorate reducing bacteria utilize O₂ in preference to perchlorate. For
example 2 mg l\(^{-1}\) oxygen concentration could completely inhibit the perchlorate reduction in *A. suillum* (Chaudhuri et al. 2002). In our study, oxygen may have leaked into the system during sampling and likely inhibited the autotrophic perchlorate reduction, although it was not measured.

Nitrate, on the other hand, is an excellent competitor of perchlorate due to a similarity in the reduction potential of the NO\(_3^-/N_2\) and ClO\(_4^-/Cl^-\) pair (1.25 V vs 1.28 V) (Bardiya & Bae 2011). This competitive inhibitory effect is reported extensively both for autotrophic (London et al. 2011) and heterotrophic processes (Wang et al. 2012).

Increasing perchlorate concentrations led to increased reduction rates, which should be due to increasing substrate availability. Increasing influent perchlorate concentration from 100 \(\mu\)g l\(^{-1}\) to 1,000 \(\mu\)g l\(^{-1}\) increased the removal rate from 1,163 \(\mu\)g l\(^{-1}\) d\(^{-1}\) to 11,600 \(\mu\)g l\(^{-1}\) d\(^{-1}\), respectively, in the autotrophic reactor. A similar \(S^0\) oxidizing reactor was reported to be treating approximately 8,000 mg l\(^{-1}\) perchlorate with a reduction rate of 13,846 \(\mu\)g l\(^{-1}\) d\(^{-1}\) (Sahu et al. 2009). The perchlorate reduction rates increased with increasing perchlorate concentrations in accordance with similar studies (Nor et al. 2011). Hence, autotrophic perchlorate reduction is a practical option to treat high concentrations of perchlorate. Mixotrophic reduction of perchlorate is more stable and efficient. Complete reduction of 1,000 \(\mu\)g l\(^{-1}\) perchlorate together with 25 mg l\(^{-1}\) NO\(_3^-\)-N was accomplished in our study with the highest perchlorate reduction rate of 16,000 \(\mu\)g l\(^{-1}\) d\(^{-1}\). Higher perchlorate reductions up to 250 mg l\(^{-1}\) were reported for heterotrophic processes (Fox et al. 2014). In our study, maximum perchlorate concentration fed to the bioreactor was 1,000 \(\mu\)g l\(^{-1}\) to simulate contaminated drinking waters. Chung et al. (2007a) evaluated perchlorate reduction in a hydrogen based membrane biofilm reactor and reported the maximum perchlorate reduction rate as 700 \(\mu\)g l\(^{-1}\) d\(^{-1}\). Wang et al. (2015) evaluated the performances of hydrogen and acetate and they found that 15 days and 8 days were required, respectively, for the reduction of 2.5 mg l\(^{-1}\) perchlorate. Nerenberg et al. (2006) obtained the specific reduction rates for perchlorate and chloride as 3,100 \(\mu\)g/(mgDW.d) and 6,300 \(\mu\)g/(mgDW.d) respectively. Drinking water standards of WHO, EU or US-EPA do not include any reference concentrations for perchlorate (Blake et al. 2009). Only EPA set a reference dose of 0.7 \(\mu\)g perchlorate/kg body weight corresponding to the drinking water equivalent level of 24.5 \(\mu\)g l\(^{-1}\), assuming water is the only source of perchlorate consumption. The autotrophic reactor provided the necessary treatment to meet with this reference dose except during the last period with 1,000 \(\mu\)g l\(^{-1}\) influent. The mixotrophic reactor gave better performance compared to the autotrophic reactor.

**Nitrate reduction**

The autotrophic reactor was operated in the absence of perchlorate for the first 312 days (period 1). In this period, nitrate loading was 50 mg NO\(_3^-\)-N l\(^{-1}\)d\(^{-1}\) and nitrate was reduced completely except for days 182–193 when operational problems occurred. For the rest of this period, effluent nitrate concentrations were below detection limits (<0.1 mg l\(^{-1}\)). In periods 2 and 3, nitrate loading was increased to 150 mg NO\(_3^-\)-N l\(^{-1}\)d\(^{-1}\) and 300 mg NO\(_3^-\)-N l\(^{-1}\)d\(^{-1}\), respectively. In these periods, effluent nitrate concentrations were below 2 mg l\(^{-1}\) NO\(_3^-\)-N. For the periods 2 and 3, average effluent nitrate concentrations were 0.33 ± 0.7 and 0.54 ± 1.1 mg NO\(_3^-\)-N l\(^{-1}\). Effluent nitrate concentrations were below detection limits in these periods except day 300 with a concentration of 0.04 mg NO\(_3^-\)-N l\(^{-1}\) (Figure 2).

Perchlorate (50 \(\mu\)g l\(^{-1}\)) was added to the feed in period 4 (day 316) and its concentration was increased gradually to 1,000 \(\mu\)g l\(^{-1}\) (Table 1) at a constant nitrate loading rate of around 300 mg NO\(_3^-\)-N l\(^{-1}\)d\(^{-1}\). The addition of perchlorate did not affect the denitrification performance adversely (Figure 2). In periods 4 and 5, when influent perchlorate was 50 \(\mu\)g l\(^{-1}\) and 100 \(\mu\)g l\(^{-1}\), effluent nitrate concentrations averaged 0.41 ± 1.48 mg NO\(_3^-\)-N l\(^{-1}\) and 0.07 ± 0.48 mg NO\(_3^-\)-N l\(^{-1}\), respectively. Influent perchlorate concentrations were further increased to 200, 300, 500 and 1,000 \(\mu\)g l\(^{-1}\) in the following periods, however denitrification efficiency was not adversely affected. Throughout the study, effluent nitrate concentration averaged 0.22 ± 0.8 mg NO\(_3^-\)-N l\(^{-1}\) (ignoring days 182–193, during which operational problems occurred). In periods 3–9, nitrate reduction rates were around 300 mg l\(^{-1}\)d\(^{-1}\), which is comparable with the performance of a similar reactor in which nitrate and chromate were simultaneously reduced and the maximum denitrification rate was 500 mg l\(^{-1}\)d\(^{-1}\) (Sahinkaya et al. 2015). Sahinkaya & Dursun (2012) also obtained the
denitrification rates of 450 mg l\(^{-1}\) d\(^{-1}\) and 300 mg l\(^{-1}\) d\(^{-1}\) for mixotrophic and autotrophic processes respectively.

The mixotrophic reactor was operated for 174 days under 9 different operational periods (Table 1). In the first period, the nitrate load was 50 mg l\(^{-1}\) d\(^{-1}\) and it increased to 150 mg l\(^{-1}\) d\(^{-1}\) and then 400 mg l\(^{-1}\) d\(^{-1}\) in period 2 and 3 respectively (Figure 3). Complete nitrate reduction (25 mg NO\(_3^-\)N l\(^{-1}\)) was achieved in the first period. When the nitrate load was increased to 150 mg l\(^{-1}\) d\(^{-1}\), effluent nitrate concentration was almost under the detectable level, however nitrite (<1 mg l\(^{-1}\)) was detected for the first three days of period 2. In the third period, the reactor was operated under mixotrophic conditions and heterotrophic denitrification was stimulated with the addition of methanol to the feed at 25 mg l\(^{-1}\). The fraction of nitrate denitrified by heterotrophs was determined indirectly from the production of sulfate (Oh et al. 2001). According to Equation (1), 2.47 mg methanol is required to reduce each milligram of NO\(_3^-\)N corresponding to around 10 mg NO\(_3^-\)N to be reduced heterotrophically. Thus a decrease in sulfate production of 75.4 mg was theoretically expected since 7.54 mg SO\(_4^{2-}\) is produced for each mg NO\(_3^-\)N reduced autotrophically. Average sulfate concentration decreased from 238 ± 36.40 to 176.10 ± 11.50 mg l\(^{-1}\), corresponding to around 8 mg l\(^{-1}\) NO\(_3^-\)N reduction heterotrophically. Similarly, when methanol in the feed was further increased to 35 mg l\(^{-1}\) in period 8, effluent sulfate concentration decreased to 142.10 ± 20.90 mg l\(^{-1}\) corresponding to an increase of heterotrophically reduced NO\(_3^-\)N concentration from 8 to 12.5 mg l\(^{-1}\). Throughout the study, average
effluent nitrate concentration was 0.20 ± 0.9 mg NO$_3^-$-N l$^{-1}$. Complete reduction of 25 mg NO$_3^-$-N l$^{-1}$ was achieved and the maximum nitrate reduction rate for autotrophic and mixotrophic reactors were 300 mg NO$_3^-$-N l$^{-1}$d$^{-1}$ and 400 mg NO$_3^-$-N l$^{-1}$d$^{-1}$, respectively. Batch adsorption studies revealed that ClO$_4^-$ and NO$_3^-$ were not adsorbed on elemental sulfur/limestone for both reactors (data not shown). The mixotrophic reactor was operated under methanol limiting conditions as methanol/NO$_3^-$-N ratio in the influent was 1–1.4 mg methanol/mg NO$_3^-$-N. In the reactor, average methanol utilization per milligram heterotrophically removed nitrate was 2.73 ± 1.75 in periods 3–9. According to reaction 2, theoretically 2.47 mg CH$_3$OH is required to denitrify each milligram of NO$_3^-$-N. However, a generally higher methanol requirement has been reported, which may be due to higher biomass generation compared to Reaction 2. The methanol requirement for heterotrophic denitrification was reported as 2.65 ± 0.3 mg methanol/mg NO$_3^-$-N in the study of Sahinkaya & Kilic (2014) (Sahinkaya & Kilic 2014). Similarly 2.72 (Sahinkaya et al. 2011) and 3.06 (Sahinkaya et al. 2013) were the other reported methanol/NO$_3^-$-N ratios in denitrifying reactors.

**Sulfate production**

According to Equation (2), in the autotrophic process 7.54 mg sulfate is produced for each mg NO$_3^-$-N reduced. Influent and effluent sulfate concentrations together with the theoretical sulfate concentration are presented in Figure 2. Average theoretical effluent sulfate concentration was 215 ± 25.60 mg l$^{-1}$,
whereas effluent sulfate concentration of the autotrophic reactor averaged 259 ± 87.70 mg l⁻¹ throughout the study. The average sulfate concentration of tap water was 30 ± 10.60 mg l⁻¹. High effluent sulfate concentrations should be due to the leakage of oxygen during sampling or feeding, although some precautions (e.g. nitrogen gas bubbling of feed after preparation and keeping the feed in a collapsible container) were taken. The mixotrophic reactor was supplemented with methanol in period 4 at a concentration of 25 mg CH₃OH l⁻¹. The addition of methanol decreased average sulfate production from 238.20 ± 36.40 to 176.10 ± 11.50 mg l⁻¹. Sulfate production decreased significantly after methanol addition. The fast process response was probably due to the fact that sludge used for inoculation was taken from another mixotrophic reactor reducing nitrate. In period 8, increasing the methanol in the feed to 35 mg l⁻¹ led to a further decrease in sulfate concentration to 150.25 ± 16 mg l⁻¹. Considering the sulfate generation in the case of 25 mg and 35 mg methanol supplementations, it was calculated that around 67% and 53% NO₃⁻N was autotrophically denitriified, respectively. Average effluent sulfate concentration was below the US-EPA, EU, and Turkish drinking water standard of 250 mg l⁻¹. According to the results, the sulfate concentration in the effluent of the reactor can be controlled by external carbon supplementation.

Alkalinity

The effluent pH and alkalinity concentrations of the autotrophic reactor were lower than those of the influent due to acid generation in autotrophic processes (Equation (4)). While average influent alkalinity was 443 ± 62 mg CaCO₃ l⁻¹, it decreased to 215 ± 63 mg CaCO₃ l⁻¹ in the effluent. Sulfur based autotrophic denitrification of each mg NO₃⁻N would consume 4.57 mg CaCO₃. Therefore, denitrification of 25 mg NO₃⁻N would consume 114 mg CaCO₃. The average effluent alkalinity concentration can then be calculated as around 329 ± 66 mg CaCO₃ l⁻¹, which is much higher than the measured concentrations. This result indicated that acidity may also be produced due to oxidation of elemental sulfur with the oxygen leaking to the reactor with feed or during its operation. The average influent pH of 8.1 ± 0.4 decreased down to 7.8 ± 0.6. Similar results were found in periods 3 and 4 as well. In period 4 and subsequent periods, the addition of perchlorate did not affect the effluent pH and alkalinity. The mixotrophic reactor was filled with limestone and elemental sulfur and operated for the first two periods in autotrophic mode in the absence of methanol. The effluent alkalinity averaged 129 ± 9.0 mg CaCO₃ l⁻¹ in periods 1 and 2, at influent alkalinity concentration of 125.60 ± 11.50 mg l⁻¹. In the third period, the addition of methanol promoted the heterotrophic denitrification and also alkalinity production. For periods 3–7, almost complete denitrification was achieved at 25 mg NO₃⁻N l⁻¹. Average NO₃⁻N concentration reduced by the heterotrophic process was 8.25 ± 0.24 mg l⁻¹ in periods 3–7. In the heterotrophic denitrification, theoretically 3.57 mg CaCO₃ l⁻¹ is produced for each mg NO₃⁻N denitrified (Equation (1)) (Oh et al. 2001). Similarly, 4.57 mg CaCO₃ is utilized in autotrophic denitrification of each mg NO₃⁻N. Considering that 8.25 mg NO₃⁻N is denitrified by heterotrophs, the whole system then needed 47 mg CaCO₃. Limestone dissolution was another factor providing alkalinity to the system. Influent and effluent alkalinity concentrations of periods 3–7 (influent

![Figure 4](https://iwaponline.com/ws/article-pdf/16/1/208/413262/ws016010208.pdf)
methanol concentration was 25 mg l\(^{-1}\)) were 122.50 ± 16 mg CaCO\(_3\) l\(^{-1}\) and 143.60 ± 14.60 mg CaCO\(_3\) l\(^{-1}\) respectively. When methanol was further increased to 35 mg l\(^{-1}\), influent and effluent alkalinity concentrations were 120 ± 12 and 161 ± 28 mg CaCO\(_3\) l\(^{-1}\).

**Residual organics**

Methanol as an organic carbon source was added to the mixotrophic reactor in periods 3–9. Dissolved organic carbon was almost completely removed in the bioreactor. Influent methanol concentrations were 25 mg l\(^{-1}\) and 35 mg l\(^{-1}\) in periods 3–7 and 8–9 respectively. Residual organics were measured as dissolved organic carbon and effluent DOC concentrations were below 2 mg l\(^{-1}\) (Figure 4).

Ethanol, methanol and acetic acid are the most common carbon sources used in full-scale drinking water denitrification processes (Matějů et al. 1992). However, there are some concerns regarding the utilization of these organic carbon sources for drinking water treatment. Organic carbon in water distribution systems may promote bacterial growth. In addition, by-products may build up from organic carbon sources in disinfection processes. Additionally, a poor C/N ratio leads to improper denitrification, which leads to accumulation of nitrite or extra production of nitrous other than nitrogen gas (Kim et al. 2002). A combination of elemental sulfur based autotrophic process with methanol based heterotrophic process could overcome all these drawbacks. Proper dosing of methanol to the autotrophic denitrifying reactor could completely remove nitrate and perchlorate without excess sulfate or DOC contamination in the effluent.

**CONCLUSIONS**

Complete removal of nitrate was achieved in both autotrophic and mixotrophic reactors at the loading rates of 300 mg l\(^{-1}\)d\(^{-1}\) and 400 mg l\(^{-1}\)d\(^{-1}\), respectively. Perchlorate was decreased from 1,000 to 33.23 ± 30.4 μg l\(^{-1}\) in the autotrophic reactor corresponding to 97% perchlorate removal. In the mixotrophic reactor, perchlorate was reduced completely at varying influent concentrations of 50–1,000 μg l\(^{-1}\). Effluent sulfate concentrations in the mixotrophic reactor were lower than the EU, EPA and TS266 (Turkish standard for water intended for human consumption) drinking water standards. Stimulating the mixotrophic denitrification process by methanol supplementation offered advantages of (1) higher perchlorate removal rate, (2) lower effluent sulfate concentration and (3) complete nitrate reduction under nitrate loading rates of 400 mg N l\(^{-1}\)d\(^{-1}\).

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