ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

SIMULTANEOUS NITRATE AND PERCHLORATE REDUCTION FROM DRINKING WATER BY ELEMENTAL SULFUR-BASED AUTOTROPHIC AND MIXOTROPHIC DENITRIFICATION PROCESSES

Ph.D. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

IÇME SULARINDAN NİTRAT VE PERKLORATIN KÜKÜRT BAZLI OTOTROFİK VE MİKSOTROFİK DENİTRİFİKASYON SÜREÇLERİYLE EŞZAMANLI GİDERİMİ

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OCAK 2016

Deniz UÇAR, a Ph.D. student of ITU Graduate School of Science Engineering and Technology 501102806, successfully defended the thesis/dissertation entitled "**SIMULTANEOUS NITRATE AND PERCHLORATE REDUCTION FROM DRINKING WATER BY ELEMENTAL SULFUR-BASED AUTOTROPHIC AND MIXOTROPHIC DENITRIFICATION PROCESSES**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my parents and my sister,

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ABBREVIATIONS

COD	: Chemical Oxygen Demand
VSS	: Volatile Suspended Solid
IC	: Ion Chromatography
CDD	: Conductivity Detector
SCD	: Supressed Conductivity
UAR	: Upflow Anaerobic Reactor
HRT	: Hydraulic Retention Time
SRT	: Sludge Retention Time
DOC	: Dissolved Organic Carbon
gDNA	: Genomic DNA
PCR	: Polymerase Chain Reaction
qPCR	: Quantitative Real Time PCR
US-EPA	: United States Environmental Protection Agency
WHO	: World Health Organization
EU	: European Union

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SIMULTANEOUS DENITRIFICATION AND PERCHLORATE DESTRUCTION FROM DRINKING WATER BY SULFUR-BASED AUTOTROPHIC AND MIXOTROPHIC PROCESS

SUMMARY

Perchlorate (ClO_4^-) is the salt derived from perchloric acid. Perchlorate, can contaminate drinking water sources via natural and anthropogenic sources. Perchlorate is used in products such as airbags, fireworks, military weapons (eg. Missiles and rockets) and by the pharmaceutical industries (eg. For the treatment of hyperthyroidism). It naturally forms from the chemical reaction between chlorine gas and ozone and can be found in nitrate deposits in arid regions such as the Atacama Desert in Chile. With the utilization of nitrates as fertilizer, perchlorate might be more commonly found in the groundwater together with nitrate.

Due to the high solubility and low adsorption properties of ammonium perchlorate, it can easily reach sources of drinking water. Perchlorate blocks the iodine uptake by the thyroid, decreasing the thyroid hormone (triiodothyronine-T3 and thyroxine-T4) concentrations in the body. These hormones regulate basal metabolisms in adults and normal development processes in children. Therefore the presence of perchlorate in drinking water might contribute to the onset of a variety of basal metabolic disorders.

Current remediation methods are classified into two sub-groups: removal or destruction. Some removal methods include ion exchange, membrane filtration and adsorption. Destruction methods involve chemical, electrochemical or biological reduction. Besides the additional chemical requirements for these reduction processes, these removal methods are costly, and have low efficiencies when other contaminants are present in the solution. Most seriously, this removal method leads to the discharge of concentrated brine. Regardless, biological reduction is the most common method, due to its fast reaction rate, and the fact that it does not require expensive catalyst or chemicals. In many studies, ClO_4^- is sucessfully biologically reduced to Cl^- ion. However, these widely used conventional drinking water treatment processes are not effective in the treatment of perchlorate.

In the last decade it has become possible to detect low concentrations of perchlorate, due to improvements in the analytical methods used. Since then, the presence of perchlorate has been detected in the drinking water of many countries, especially in the USA. However, perchlorate is not listed as hazardous by the standards of the World Health Organization (WHO), the European Union, TS266 (regulations on water intended on human consumption), or the US-EPA. While some US states have already established standards (Arizona, 14 μg/L; Massachusetts, 2 μg/L; Nevada, 18 μg/L), an Interim Health Advisory Level of 15 μg/L has been released by the EPA.

Nitrate is one of the most commonly encountered pollutant in surface and groundwater. The most important sources of nitrate in groundwater are nitrogen containing fertilizers and the release of improperly treated wastewater from industrial and domestic sources. According to TS266, the maximum allowed concentrations of NO_3^--N and NO_2^--N are 11.3 and 0.15 mg/L, respectively. Additionally, many sources of groundwater may be contaminated with both nitrate and ClO_4^-, necessitating the development of processes able to simultaneously remove nitrate and perchlorate. Recently, many studies on autotrophic denitrification processes have been conducted. In sulfur-based autotrophic process (SLAD: sulfur-limestone autotrophic denitrification) sulfur and nitrate are used as electron donors and acceptors, respectively. As a result, sulfur is oxidized to sulfate, and nitrate is reduced to nitrogen gas. The reaction is given below (Equation 1), and CO_2 is used as a carbon source.

$$55S^0+50\ NO_3^-+38H_2O+20CO_2+4NH_4^+ \rightarrow 4C_5H_7O_2N+55SO_4^{2-}+25N_2+64H^+ \quad (1)$$

Although sulfur-based autotrophic denitrification has several advantages, its not widely used due to sulfate and acid formation. In heterotrophic denitrification, however, sulfate is not produced and 3.57 g $CaCO_3$ is formed per gram of NO_3^--N reduced (Equation 2).

$$NO_3^-+1.08CH_3OH+0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2+0.47N_2+1.68H_2O+HCO_3^- \quad (2)$$

Even though there is no sulfate formed and the pH decreases in heterotrophic processes, these processes are highly sensitive to the quantity of (organic) electron donors. For example addition of more organic compounds may lead to effluent that still contains those compounds; whereas, nitrite formation may be observed in the case of lower amount of organic supplementation. Therefore, mixotorophic processes, that combine heterotrophic and autotrophic denitrification, can be used to control the amount of sulfate formation, the pH and the risk of residual organic compounds. Additionally, both nitrate and perchlorate reduction could be achieved at higher rates compared to autotrophic processes. Therefore the aims of this thesis are: (1) evaluation of sulfur-based autotrophic denitrification processes for the simultaneous reduction of nitrate and perchlorate from drinking water; (2) simultaneous reduction of both oxianions by methanol-based heterotrophic processes; (3) stimulation of the mixotrophic process by addition of organic matters to
autotrophic process for simultaneous nitrate and perchlorate reduction; (4) evaluation of heterotrophic-autotrophic sequential process for nitrate and perchlorate reduction and (5) the determination of microbial community in the bioreactors under varying operational conditions. In addition to these aims, the effect of different alkalinity sources on autotrophic reactors was investigated.

For this thesis project, two autotrophic, and one heterotrophic reactors were operated for more than 100 days. The mixotrophic reactor and the heterotrophic-autotrophic sequential system were operated for 174 and 100 days, respectively. Real groundwater polluted with nitrate was supplied to the sequential system for a 7 day period. To identify the nitrate and perchlorate removal mechanism, batch tests were conducted for 90 hours. The dominant microbial community was determined using molecular tools.

The first autotrophic reactor was filled with elemental sulfur and limestone particles. The feed contained 25 mg NO_3^--N /L and various concentrations of perchlorate (50-1000 µg/L). Complete nitrate reduction was achieved in all tested conditions. Although high perchlorate removals were attained, perchlorate was always detected in the effluent; the concentrations varied from 21.88–85 µg/L depending on the perchlorate and nitrate loadings. Perchlorate was reduced from 1000 µg/L to 53±21.36 µg/L, corresponding to around 95% reduction with an HRT of 2 h. Simultaneous reduction of both anions produced sulfate in the effluent with 227±30 mg SO_4^{2-}/L. The average influent alkalinity concentration was decreased from 155±23 to 96±30 mg $CaCO_3$/L but not below this threshold because of the limestone. In the second autotrophic reactor, $NaHCO_3$ was added to compare the effect of alkalinity sources. This reactor also highly reduced perchlorate and decreased perchlorate from 1000 µg/L to 33.23±30.4 µg/L. The influent nitrate concentration of 25 mg NO_3^--N/L was completely reduced. The effluent sulfate concentration of the autotrophic reactor averaged 259±87.70 mg/L throughout the study. For autotrophic reactors the maximum reduction rate of nitrate was 300 mg NO_3^--N/(L.d). The heterotrophic process reduced nitrate and perchlorate completely in all tested conditions (i.e., 25 mg NO_3^--N/L and 50-1000 µg ClO_4^-/L). However, 2 – 4 mg/L DOC was detected in the effluent.

The complete reduction of 25 mg NO_3^--N/L and 1000 µg/L perchlorate was also accomplished in mixotrophic reactor and the effluent sulfate concentration was controlled by adjusting the C/N ratio in the influent. Mixotrophic denitrification was stimulated by the addition of 25 mg/L methanol. Fifty-three percent, of influent nitrate was reduced by heterotrophic process, which decreased the effluent sulfate concentration to half of the autotrophic counterpart. Effluent DOC concentrations were below 2 mg/L.

Lastly a heterotrophic-autotrophic sequential system was investigated for the reduction of both anions. Heterotrophic effluent was pumped to autotrophic reactor. In this configuration, nitrate and perchlorate were reduced completely with maximum initial concentrations of 100 mg NO_3^--N/L and 1000 µg/L respectively. The C/N ratio varied between 1.24 and 2.77 throughout the study, effluent sulfate concentration was below the drinking water standard of 250 mg/L and the pH was neutral.

İÇME SULARINDAN NİTRAT VE PERKLORATIN KÜKÜRT BAZLI OTOTROFİK VE MİKSOTROFİK SÜREÇLERLE EŞZAMANLI GİDERİMİ

ÖZET

Perklorat (ClO_4^-), perklorik asitten elde edilen tuzlardır. Doğal ve antropojenik kaynaklardan içme sularına karışabilen perklorat, hava yastığı, havai fişek gibi bazı ürünlerde, füze – roket gibi askeri silahlarda ve bazı ilaç endüstrilerinde (hipertiroidizm tedavisinde) kullanılmaktadır. Perklorat, doğal yollardan ise atmosferde ozon ile klor gazlarının reaksiyonu neticesinde oluşmaktadır. Ayrıca ClO_4^- kurak bölgelerde bulunan nitrat yataklarında da bulunabilmektedir (Atacama Çölü/Şili). Bu bölgelerdeki nitratın gübre olarak kullanılmasıyla ClO_4^- yeraltı sularında nitrat ile birlikte bulunabilmektedir.

Amonyum perklorat, sudaki yüksek çözünürlüğü ve düşük adsorpsiyon eğilimi nedeniyle içme sularına kolaylıkla karışabilmektedir. Perklorat, tiroit bezlerinin iyot bağlamasını engelleyerek vücuttaki tiroit hormonlarının (triiyodotironin-T3 ve tiroksin-T4) konsantrasyonunu düşürmektedir. Bu hormonlar yetişkinlerde bazal metabolizmayı, çocuklarda ise büyüme ve gelişmeyi düzenlerler. Bu nedenle içme sularında perklorat varlığı, bazal metabolizmaya bağlı birçok hastalığı tetiklemektedir.

İçme sularından perklorat giderilmesinde kullanılan mevcut yöntemler giderim ve parçalama olarak ikiye ayrılmaktadır. Giderim yöntemleri, iyon değişimi, membran filtrasyonu ve adsorpsiyon iken, parçalama yöntemleri kimyasal, elektrokimyasal ve biyolojik indirgemedir. Giderim yöntemleri, ilave kimyasal ihtiyaçları, yüksek maliyetleri ve başka kirleticilerin ortamda bulunması durumunda düşük verim göstermelerinin yanında deşarj edilmesi gereken konsantre atıksu oluşturmaları gibi ciddi dezavantajlara sahiptirler. Parçalama yöntemlerinde ise biyolojik indirgeme, yüksek reaksiyon hızı, pahalı katalizörlere ya da kimyasallara ihtiyaç duymaması gibi nedenlerle en çok kullanılan yöntemdir. Birçok çalışmada ClO_4^- biyolojik olarak başarılı bir şekilde zararsız Cl^- iyonununa indirgenmiştir. Ülkemizde yaygın olarak bulunan konvansiyonel içme suyu arıtma prosesleri perklorat gideriminde etkili değildir.

Düşük konsantrasyonlardaki perklorat ölçümü son on yılda gelişen analitik yöntemlerle mümkün olmuştur. Buna bağlı olarak başta ABD olmak üzere birçok ülkede içme sularında perklorat varlığı tespit edilmiştir. Ancak perklorat henüz Dünya Sağlık Örgütü (WHO), Avrupa Birliği, TS266 (insani tüketim amaçlı sular yönetmeliği) ve EPA'ya ait içme suyu standartlarında yer almamaktadır. bu ABD'de bulunan bazı eyaletler nedenle kendilerine ait standartları belirlerken (Arizona, 14 µg/L; Massachusetts, 2 µg/L; Nevada, 18 µg/L), EPA geçici limiti 15 µg/L olarak bildirmiştir.

Nitrat, yüzeysel ve yeraltı sularında en çok karşılaşılan kirleticilerden biri olup, en önemli kaynakları tarımsal gübre kullanımı ve ileri arıtma yapılmadan deşarj edilen evsel ve endüstriyel nitelikli atıksulardır. TS 266'ya göre içme suyunda nitrat ve nitrit için sınır değerler sırasıyla 11,3 mg NO_3^--N/L ve 0,15 mg NO_2^--N/L'dir. Birçok yeraltı suyunda nitrat ve perklorat birlikte bulunabilmektedir. Dolayısıyla eşzamanlı olarak nitrat ve perklorat giderimi yapabilen biyolojik sistemlerin geliştirilmesine ihtiyaç duyulmaktadır. Son zamanlarda, içme sularından nitrat giderimi için ototrofik biyolojik denitrifikasyon prosesleri üzerine yoğun çalışmalar yapılmaktadır. Kükürt bazlı ototrofik denitrifikasyon prosesinde (SLAD: sulfur-limestone autotrophic denitrification) *Thiobacillus denitrificans* ve *Thiomicrospira denitrican* gibi türler görev alarak, kükürtü elektron verici, nitrat ve nitriti de elektron alıcı olarak kullanırlar. Proses sonunda kükürt sülfata, nitrat ise azot gazına dönüşür. Reaksiyon basit olarak aşağıda gösterilmiş olup, karbon kaynağı olarak CO_2 kullanılmaktadır.

$$55S^0+50NO_3^-+38H_2O+20CO_2+4NH_4^+ \rightarrow 4C_5H_7O_2N+55SO_4^{2-}+25N_2+64H^+ \quad (1)$$

Sülfür bazlı ototrofik denitrifikasyon sisteminin birçok avantajı olmasına rağmen, esas dezavantajları sülfat ve asidite üretmesidir. Fakat heterotrofik denitrifikasyonda sülfat üretilmezken indirgenen g NO_3^--N başına 3.57 g $CaCO_3$ üretilir.

$$NO_3^-+1.08CH_3OH+0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2+0.47N_2+1.68H_2O+HCO_3^- \quad (2)$$

Heterotrofik süreçlerde ise sülfat üretimi ve pH'da düşüş olmamasına rağmen, elektron kaynağının net ayarlanması esas dezavantajdır. Yüksek organik madde eklenmesi çıkışta kalıntı organik madde kalmasına sebep olabilirken, düşük organik madde yüklenmesi de çıkış suyunda nitrit gözlemlenmesine sebep olabilir. Bu nedenle, heterotrofik ve ototrofik süreçlerin kombinasyonu ile oluşturulan mixotrofik sistemler ile çıkış sülfat, organik kalıntı riski ve pH sorunu kontrol altına alınabilinir. İlave olarak hem nitrat hem de perklorat, ototrofik süreçlere nazaran daha yüksek seviyelerde indirgenebilir. Bu bağlamda bu tezin amaçları (1) içme sularından nitrat ve perkloratın birlikte giderimi için kükürt bazlı ototrofik denitrifikasyon süreçlerinin incelenmesi, (2) her iki anyonun metanol bazlı heterotrofik süreçlerde giderilmesi ve bu sürecin ototrofik sistemler ile mukayesesi. (3) metanolün ototrofik reaktöre eklenerek miksotrofik bir süreç oluşturulması ve avantajlarının belirlenmesi, (4) hem heterotrofik hem de ototrofik süreçlerin daha iyi izlenebilmesi için heterotrofik – ototrofik bir sıralı sistemin incelenmesi ve (5) Reaktörlerdeki baskın mikrobiyal kominitenin tespitidir. İlave olarak ototrofik reaktörlerde farklı alkalinite kaynakları kullanılarak bunun giderim verimine olası etkisi araştırılmıştır.

Bu tez projesinde, 2 adet ototrofik ve 1 adet heterotrofik reaktör 100 den fazla gün boyunca işletilmiştir. Miksotrofik reaktör 174 gün, heterotrofik – ototrofik sıralı sistem ise 100 gün boyunca işletilmiştir. Ek olarak nitrat kirliliği bulunan bir gerçek yeraltı suyunun arıtılabilirliği 7 günlük bir periyotta sıralı sistemde test edilmiştir. Nitrat ve perkloratın giderim mekanizmalarının belirlenebilmesi içinde 90 saatlik bir kesikli test yapılmıştır. Son olarak baskın kominite yapılarının belirlenmesi için ise moleküler teknikler kullanılmıştır.

Çalışmada ilk ototrofik reaktör elementel kükürt ve kireçtaşı partikülleri ile doldurulmuştur. Giriş suyu 25 mg NO_3^--N/L ve çeşitli konsantrasyonlarda (50-1000 µg/L) perklorat içermiştir. Test edilen tüm koşullarda nitrat tamamen giderilmiştir.

Yüksek seviyelerde perklorat indirgenmiş olmasına rağmen, çıkış suyunda 21.88–85 µg/L arasında değişen konsantrasyonlarda perklorat tespit edilmiştir. Perklorat 1000 µg/L'den 53±21.36 µg/L'ye düşürülerek 2 saatlik HRT'de %95'lik bir giderim verimi sağlanmıştır. Her iki anyonun birlikte giderimi çıkış suyunda 227±30 mg SO_4^{2-}/L oluşumuna sebep olmuştur. Ortalama giriş alkalinite konsantrasyonu 155±23 to 96±30 mg $CaCO_3$/L seviyelerine inmiş ancak kireçtaşı sayesinde daha fazla düşme eğilimi göstermemiştir. İkinci ototrofik reaktörde, alkalinite kaynağının etkisinin kıyaslanabilmesi için $NaHCO_3$ kullanılmıştır. Bu reaktörde de yüksek perklorat giderim verimi gözlenmiş ve perklorat 1000 µg/L 'den 33.23±30.4 µg/L'ye indirgenmiştir. Giriş 25 mg NO_3^--N/L tamamen giderilmiştir. Bu ototrofik reaktöre ait çıkış sülfat konsantrasyonu tüm çalışma boyunca ortalama 259±87.70 mg/L'dir. Her iki ototrofik reaktör için maksimum nitrat giderim oranı 300 mg NO_3^--N/(L.d) 'dir. Heterotrofik reaktör ise hem perkloratı hemde nitratı test edilen tüm koşullarda tamamen giderirken 2 – 4 mg/L DOC çıkış suyunda tespit edilmiştir.

25 mg NO_3^--N/L and 1000 µg/L perkloratın tamamı miksotrofik reaktörde giderilmiş ve çıkış sülfat konsantrasyonu C/N oranının ayarlanması ile kontrol altında tutulmuştur. Miksotrofik reaktör 25 mg/L methanol eklenmesi ile sağlanmış ve giriş nitratın %53'ü heterotrofik proseste giderilmiştir. Bu sayede çıkış sülfat konsantrasyonu ototrofik kısma göre yarı yarıya azalmıştır. Çıkış DOC konsantrasyonuda 2 mg/L'nin altında gözlemlenmiştir.

Son olarak heterotrofik ototrofik sıralı sitem her iki anyonun giderilmesi için araştırılmıştır. Heterotrofik çıkış suyu ototrofik reaktöre verilmiştir. Bu düzenekte 100 mg NO_3^--N/L and 1000 µg/L perklorat tamamen giderilmiştir. C/N oranı 1.24 ile 2.77 arasında tutulmuş ve çıkış sülfat konsantrasyonu içme suyu standart değeri olan 250 mg/L'nin altına indirilmiş, pH nötral seviyelerde kalmıştır.

1. INTRODUCTION

Perchlorate is a naturally occurring and man-made chemical that is used to produce rocket fuel, fireworks, flares and explosives. Perchlorate can also be present in bleach and in some fertilizers. The most common form of the perchlorate ion is the ammonium perchlorate (NH_4ClO_4) salt, which is widely used as an oxidant in rocket and missile propellants. Perchlorate salts are also used in ammunition, fireworks, flares, and a variety of other industrial products and processes (Gullick, Lechevallier, & Barhorst, 2001). Perchlorate manufacturing and the improper disposal of perchlorate containing propellants and other materials have led to widespread soil and groundwater contamination.

Figure 1.1 : Perchlorate is used in the missile systems in the Turkish Army. The combustible materials such as boron, magnesium and zirconium are mixed with oxidants such as potassium nitrate, PTFE or potassium perchlorate by using some polymeric binders (HİSAR-A, Low range anti-aircraft missile system, developed by ASELSAN and ROKETSAN)(Dinçer, 2014).

Perchlorate is highly mobile in surface and subsurface waters since it is nonvolatile, does not sorb to soil minerals, and there is little evidence of biological transformation under natural conditions (Urbansky & Schock, 1999). Ammonium perchlorate salt solubility in water is 250,000 mg/L and has a saturated solution density of approximately 1.11 g/cm^3. Solubilities of other perchlorate salts are presented in Table 1.1. These properties of high solubility and density make predicting the migration of the brine in the subsurface a difficult task (Urbansky & Schock, 1999).

Table 1.1 : Solubility of perchlorate salts (Tikkanen, 2006).

Perchlorate Salt	Solubility, g/L (25 °C)
Ammonium Perchlorate	249
Lithium Perchlorate	597
Potassium Perchlorate	21
Sodium Perchlorate	2096

Taking high doses of perchlorate may block iodine uptake by the thyroid. This blockage has been exploited in medicine to treat patients who suffer from Graves Disease (Hunt, Sitar, & Udell, 1988). Nonetheless, the effect of perchlorate on the thyroid is of concern when the chemical is accidentally introduced into the environment. It competitively inhibit iodide from entering the thyroid by affecting the Na^+/I^- symporter. Hence, the synthesis of thyroid hormones (known as triiodothyronine-T3 and thyroxine-T4) is prevented. These hormones regulate basal metabolism in adults, and aid in the proper development of children. Consumption of large doses of perchlorate may interfere with human and animal growth and development by interfering with the normal production of thyroid hormones (Wolff, 1998).

No national drinking water standards currently exist for perchlorate in the European Union (EU), USA, Canada or Australia, nor is there a guideline set by the World Health Organization. The United States pays more attention to perchlorate contamination than other countries. Although there were significant discussions between Congress, the EPA and some other authorities in the US (The House Energy and Commerce Environment, Hazardous Materials Subcommittee) over perchlorate regulation, US-EPA concluded in October of 2008 that the perchlorate levels in over

99% of drinking water posed no risk to public health and recommended that perchlorate in drinking water not be regulated at a national level (Watch, 2008). Some believed that the decision was due to the fact that treatment of perchlorate on the basis of regulation would cost millions of dollars (Eilperin, 2008). Currently perchlorate is on the EPA's Candidate Contaminants List (US EPA, 2009). However, some states in the USA have already developed limit values for perchlorate. A recent National Academy of Science (NAS) report on the potential health effects of perchlorate recommended a perchlorate reference dose of 0.0007 mg/kg of body weight, equivalent to a drinking water concentration of 24.5 μg/L. California has established a Public Health Goal (PHG) of 6 μg/L for perchlorate, and a proposed drinking water regulation is imminent (Tikkanen, 2006). Table 1.2 below shows some US state limits for perchlorate in drinking water.

Table 1.2 : US state limits for perchlorate in drinking water (Srinivasan & Sorial, 2009).

State	Standard	Regulation	Year
California	6	MCL	2007
Massachusetts	2	MCL	2006
Texas	4	DWAL	2002
Arizona	14	HBGL	2003
Nevada	18	AL	2005
New Mexico	1	DWSL	2006
	5	DWPL	
New York	18	PNL	2008

MCL: Maximum contaminant level; DWAL: drinking water action level; HBGL: health based guidance level; Al: action level; DWSL: drinking water screening level; DWPL: drinking water planning level; PNL: public notification level.

Nitrate is a common pollutant of groundwater in many countries. Due to its toxicity and its widespread presence, removal alternatives have been investigated by many local and national authorities as well as researchers. The utilization of fertilizers (Su & Puls, 2004) containing nitrogen and municipal wastewaters (Soares, 2000) are the main sources of the pollution. According to reports of EU groundwater pollution monitoring stations, 20% of wells shows over 50 mg NO_3^-/L and 40% showed 25 mg NO_3^-/L from 1996 – 1998. In developing countries, nitrate concentrations are much higher (Della Rocca, Belgiorno, & Meriç, 2007). Some wells in the Harran Plain in

Sanliurfa, Turkey have nitrate concentrations as high as 180 mg NO_3^--N/L; the average concentration of whole plain was 35 mg NO_3^--N/L (Yesilnacar, Sahinkaya, Naz, & Ozkaya, 2008).

Nitrate causes methemoglobinemia, a disorder characterized by the presence of higher than normal levels of methemoglobin (metHb, i.e., ferric [Fe^{3+}] rather than ferrous [Fe^{2+}] haemoglobin) in the blood (Park, Kim, Choi, & Pak, 2005). Methemoglobin forms in the presence of nitrate and is a form of hemoglobin that contains ferric [Fe^{3+}] iron and has a decreased ability to bind oxygen (Equation 1.1).

$$\text{Haemoglobin } (Fe^{2+}) + NO_3^- \longrightarrow \text{Methaemoglobin } (Fe^{3+}) \qquad (1.1)$$

When methaemoglobin forms in an infant's blood, less oxygen is carried leading to oxygen deficiency in the tissues, called blue-baby syndrome from the observable bruising and tarnishing in the skin of these infants.

Unlike perchlorate, many countries have already determined concentration limits for nitrate. In Table 1.3, nitrate and nitrite levels for several countries/organizations are presented. TS266 (Turkish Standards for Water Intended for Human Consumption) and the US-EPA recommend maximum contaminant levels of 10 mg/L NO_3^--N and 1.0 mg/L NO_2^--N for drinking water (Keskin, 2010; K. C. Lee & Rittmann, 2002).

Nitrate is a common co-contaminant with perchlorate and many studies focused on the treatment of both anions (Bardiya & Bae, 2011; Jinwook Chung, Rittmann, Wright, & Bowman, 2007). One explanation for the presence of both contaminants together is that the nitrate deposits in Chile, which have been used as fertilizers in the United States for over a century, also contain perchlorate (Srinivasan & Sorial, 2009).

Perchlorate and nitrate are not removed by conventional coagulation or water filtration treatment processes. Additionally, they are poorly absorbed by activated carbon. Physical removal methods such as ion exchange, reverse osmosis, membrane filtration and enhanced activated carbon treatment technologies have been developed and new adsorption technologies continue to be researched (Gu, Brown, Maya, Lance, & Moyer, 2001; Srinivasan & Sorial, 2009). However, some of these

technologies are not cost effective and produce concentrated wastewater with high concentrations of perchlorate. Alternative and complementary technologies are needed in water treatment plants to remove these anions.

Table 1.3 : Nitrate and nitrite limits for several countries and organizations.

		Nitrite (mg/L)	Nitrate (mg/L)
US	MCLG[a]	3.28	44.43
	MCL[b]	3.28	44.43
EEC(1998)	MCL[c]	0.5	50
EDWTP[d]		0.1	
WHO (2003)[e]		3	50
Pakistan		-	45
Australia		0.01	45
Morocco[f]		0.1	50
Argentina		0.1	45
Korea		-	44.43
Malaysia		0.005	45
Canada[g]		3.2	45
Ethiopia		3	50
IBWA[h]		1	10

[a]Guide maximum contaminant level.

[b]Maximum contaminant level.

[c]Must be respected according to equation of -

$$\frac{NO_3^-}{50} + \frac{NO_2^-}{3} \leq 1$$

[d]Effluent of drinking water treatment plant.

[e]Guideline limit (GL).

[f]Guide limit applied by ONEP, Rabat Drinking Water Treatment Plant Authority.

[g]45 mg/l is referred to the sum between nitrate and nitrite when nitrite cannot overcome 3.2 mg/l.

[h]International Bottled Water Association

Biological treatment methods are the most cost effective processes for the reduction of these anions and their final removal (Bardiya & Bae, 2011). In biological treatment, the process is called heterotrophic when an organic substrate is used. In the case where inorganic substrates are used, such as Fe^0, H_2 or S^0, the process is called autotrophic. Both systems have advantages and drawbacks and it seems like there is no comprehensive study that combines these systems to remove nitrate and perchlorate simultaneously. Therefore the aim of this thesis is the simultaneous denitrification and destruction of perchlorate from drinking water. To do this, sulfur-based autotrophic, methanol-based heterotrophic and mixotrophic processes were operated.

1.1 Purpose of Thesis

The main aim of this thesis is to remove perchlorate together with nitrate using sulfur-based autotrophic and mixotrophic denitrification processes. Although heterotrophic denitrification has a high nitrate reduction rate, added excess amounts of organic electron donors may remain in the effluent and sometimes may result in growth of sulfate reducing species. Both cases are unwanted in drinking water supplies. On the other hand, sulfur-based autotrophic denitrification does not require external organic supplementation, whereas, autotrophic denitrification may cause sulfate and acid generation. Mixotrophic denitrification combines the advantages and eliminates the disadvantages of both processes. There were five specific objectives of the study. First, I evaluated sulfur-based autotrophic denitrification processes for the simultaneous reduction of nitrate and perchlorate from drinking water. The impact of perchlorate on nitrate reduction efficiency was examined under varying operational conditions in column reactors, where lentil shape sulfur particles were used as an electron source and as an attachment medium. Next, I investigated the simultaneous nitrate and perchlorate reduction in a methanol-based heterotrophic reactor and compared autotrophic and heterotrophic processes for groundwater treatment. Then I compared alkalinity sources for the autotrophic processes. Afterwards, I evaluated the mixotrophic process to eliminate the drawbacks of the auto-heterotrophic processes and increase the rate of nitrate and perchlorate reduction. Later I analyzed simultaneous nitrate and perchlorate reduction in a heterotrophic-autotrophic sequential process. Finally, I determined the dominant bacterial communities in the reactors by using the molecular biological techniques (DNA extraction, PCR, DGGE and sequencing).

1.2 Literature Review

Sources of perchlorate are divided into two categories: anthropogenic and natural. The most famous natural source of perchlorate is the nitrate deposits in the Atacama desert in Chili (Catling et al., 2010). High concentrations of perchlorate were detected in these deposits. Isotopic evidence indicates that the perchlorate in the Atacama Desert is of atmospheric origin (Bao & Gu, 2004). Trace amounts of perchlorate are also found in some natural materials such as Chilean saltpeter, kelp, fishmeal, hanksite, potash ore (sylvinite), and playa crust (Charnley, 2008; Orris,

Harvey, Tsui, & Eldrige, 2003). It is assumed that anions such as CrO_4^{2-}, IO_3^-, $Cr_2O_7^{2-}$, NO_3^-, Cl^- and ClO_4^- are known to form and accumulate under arid conditions (Rajagopalan et al., 2006). For instance, the evaporites in the southwest USA contain perchlorate as well as other anions. The Tuzgolu basin in Central Anatolia is one of the the most arid parts of Turkey. Considering that the evaporites in the Tuzgolu basin contain above mentioned natural materials and that it is the most arid part of Turkey, it is possible that this region is also highly enriched in atmosperic originated perchlorate. To the best of the author's knowledge, perchlorate contamination in groundwater or its presence on the ground has not been monitored in such regions. In a study conducted by Catling et al. (2010), natural perchlorate production was associated with the oxidation of chlorine species through pathways involving ozone or its photochemical products in arid environments (Catling et al., 2010). The only study on perchlorate contamination in Turkey was conducted by Sungur et al. (2011 – 2012) in Hatay. Perchlorate in the range of 0.31±0.1 – 0.97±0.2 µg/L was detected in the drinking water. Similar concentrations of perchlorate were also found in irrigation water (between 0.37±0.1 – 1.06±0.2). It is proven that perchlorate is also present in milk, fish, vegetable, fruit and soil samples(A. Sungur & Sangün, 2011; Ş. Sungur & Atan, 2012).

Detection of perchlorate in such low concentrations became possible with the development of new chromatographic methods (Motzer, 2001). Using this method, perchlorate was separated from other anions by an anion exchange column and suppressed conductivity was utilized to quantify perchlorate concentrations down to 4 µg/l. After the development of this method, researchers and local authorities began to monitor perchlorate contamination and it was found that the extent of the pollution was underestimated. Perchlorate was found in several matrixes such as milk, crops and rice (Srinivasan & Sorial, 2009).

The majority of studies in the literature on biological perchlorate reduction have investigated the treatment of drinking water and groundwater contaminated with relatively low concentrations of perchlorate. Influent perchlorate concentrations examined in these studies were typically less than 1000 µg/L (Ye, You, Yao, & Su, 2012). Table 1.4 below summarizes the concentrations of perchlorate in different regions.

Table 1.4 : Perchlorate concentrations in different regions (Ye et al., 2012).

Study Locations	**Findings**
India	0.02–0.74 μg/L (mean 0.28 μg/L)
China	<0.02 to 54.4 μg/L (mean 2.20±6.39 μg/L)
California (1999)	33 samples of the 110 monitored wells had perchlorate concentration greater than 18 μg/L. (Highest concentration is 280 μg/L).
Middle Rio Grande Basin Across the coterminous United States	0.12–1.8 μg/L. 28 samples contained between 10.4 and 1000 μg/L.

Since in most groundwater perchlorate is present along with other anions such as nitrate, it is important to understand how microbial perchlorate reduction occurs in the presence of other anions. Nitrate is the most frequently encountered pollutant in groundwater and may be present with nitrate. As a result, many studies have focused on the treatment of both anions.

A significant amount of research in the last decade has been performed aiming to evaluate treatment alternatives for perchlorate and nitrate remediation in drinking water. The major removal technologies are adsorption with activated carbon (Parette & Cannon, 2005), ion-exchange (Gu, Brown, & Chiang, 2007) and membrane technologies (Huq et al., 2007). Destruction could be done by a reduction mechanisms, including biological reduction (Logan & LaPoint, 2002; Sahu, Conneely, Nüsslein, & Ergas, 2009), chemical reduction (Hurley & Shapley, 2007) and electrochemical reduction (C. Lee et al., 2011).

Microbial perchlorate reduction has shown a lot of promise for large-scale applications. Several microorganisms have been identified to successfully reduce perchlorate to chloride. Since this technology is now well established, it has been successfully demonstrated in a number of full-scale studies and has undergone significant optimization. The technology is based on the principle that certain bacteria contain special enzymes that lower the activation energy required for

perchlorate reduction, effectively using the perchlorate ion as an oxidant for their metabolism.

Depending on the electron donor, perchlorate can be removed both by autotrophic and heterotrophic microorganisms. Autotrophic microorganisms use either hydrogen gas (H_2), elemental sulfur or reduced iron. Heterotrophic microorganisms use ethanol, acetate, lactate, propionate, citrate and succinate as electron donors(Bardiya & Bae, 2011). Both groups of microorganisms and their reduction mechanisms explained in detail in the following parts of this chapter.

A small portion of the microorganisms in the nature could be propagatedas pure culture and this is an important limitation for traditional classification methods. It is reported that only 0.1 - 10% of microorganisms could be multiplied by environmental samples (Muyzer, De Waal, & Uitterlinden, 1993). Therefore *in-situ* identification is necessary to obtain a real population distribution even for species which cannot grow in medium. Developing molecular microbiology techniques in recent years, allow direct application to samples. By this way, species which cannot be cultivated could also be identified (Santegoeds et al., 1999). Therefore, molecular microbiology techniques are used extensively along with other identification techniques such as morphological, physical and biochemical tests (Juretschko et al., 1998). In order to identify microorganisms in environmental engineering studies, *in situ* hybridization (FISH), slot blot hybridization, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), cloning and DNA sequence analysis are utilized in different configuration (Delbès, Godon, & Moletta, 1998; Zumstein, Moletta, & Godon, 2000) In parallel to the developments in DNA purification, amplification and sequence analysis, it is established that 16S and 23S rRNA could be used to evolutionary markers in the result of the sequence analysis of rRNA gene. The 16S rRNA often preferred in molecular techniques since it exposed to very limited mutation. Hence, especially in last 15 years, rRNA/DNA based molecular methods, has become an important methods in the determination of genetic diversity of complex microbial populations (Rastogi & Sani, 2011). With the increasing 16S rRNA sequences in nucleic acid database, 16S rRNA based molecular microbiology techniques has become useful methods in the identification of microorganisms and microbial diversity.

The denaturant gradient gel electrophoresis (DGGE) method is an effective method in the determination of polymorphism and single base differentiates in the DNA which is cloned and amplified by PCR. Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) are forms of electrophoresis which use either a temperature or chemical gradient to denature the sample as it moves across an acrylamide gel. TGGE and DGGE can be applied to nucleic acids such as DNA and RNA, and (less commonly) proteins. TGGE relies on temperature dependent changes in structure to separate nucleic acids. DGGE was the original technique, and TGGE a refinement of it. Although the identification of microorganisms by molecular techniques are fast, practical and more realistic compared to other techniques, studies on the activity of species and their quantification are in different extent. In recent years, real time PCR techniques have increased its popularity in the determination of the nucleic acid's quantification and simultaneous monitoring of active genes. This technique has an important role in molecular methods due to its sensitive analysis ability and wide range of measurement spectrum.

A real-time polymerase chain reaction is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (Quantitative real-time PCR), semi-quantitatively, i.e. above/below a certain amount of DNA molecules (Semi quantitative real-time PCR) or qualitatively (Qualitative real-time PCR) (Derveaux, Vandesompele, & Hellemans, 2010).

Two common methods for the detection of PCR products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence.

As a combination of molecular ecology, techniques DGGE can be performed to determine microbial population whereas quantitative real-time PCR can be used to quantify gene copy number of enzymes, which are in charge of perchlorate reduction process. During perchlorate reduction, chlorate, chlorite and oxygen are produced with oxidation of an electron donor. In the Table.1.5 biochemical pathway of

perchlorate reduction is shown. The reduction of perchlorate to chlorate is shown in the first step and chlorate to chlorite in the second step is performed by perchlorate reductase (molybdopterin-dependent oxotransferase). Chlorite is then disintegrated to chloride and oxygen in the third step. Since perchlorate-reducing bacteria have facultative anaerobic nature, oxygen does not accumulate in the system.

Table 1.5 : Biochemical pathway of perchlorate reduction to chloride over chlorate and chlorite (Bardiya & Bae, 2011).

Oxidation state	+7	Perchlorate Reductase	+5	Perchlorate Reductase	+3	Dismutase	+1	Dismutase	−1
Anion name	Perchlorate		Chlorate		Chlorite		Hypo chlorite		Chloride
Formula	ClO_4^-		ClO_3^-		ClO_2^-		ClO^-		Cl^-
Structure									

Perchlorate reduction could be performed by nitrate reducers by nitrate reductase enzyme. Its reduction is also possible by perchlorate reducers with perchlorate reductase enzyme and other chlorate - perchlorate reducers.

Among the nitrate reducers, *Proteus mirabilis* is reported to be utilizing nitrate reductase-A and chlorate reductase-C enzymes (Oltmann, Reijnders, & Stouthamer, 1976). Also single nitrate reductase enzyme which is responsible for both nitrate and chlorate reduction is assumed to be utilized in some denitrifiers such *as R. capsulatus* and *R. sphaeroides* (Roldan, Reyes, Moreno-Vivian, & Castillo, 1994). Seperate pathways for nitrate reductase and chlorate reductase have been reported in dissimilatory chlorate reducing bacteria and chlorate reductase has recently been purified from *P.chloritidismutans* (Malmqvist et al., 1994).

Some nitrate reducers was reported to be reducing perchlorate but *D. agitate* strain CKB is the only known bacterium that cannot grow by nitrate reduction. In this strain, a single perchlorate reductase enzyme is believed to catalyze both nitrate and perchlorate reduction. The presence of separate enzymes for nitrate and perchlorate reduction was also proposed for *A. suillum* strain PS, *Azospira sp.* KJ and strain Perc1ace. Such strains show different perchlorate reduction pathways when grown on nitrate or perchlroate for extended time (Bardiya & Bae, 2011).

Azospira oryzae GR-1 which is a chlorate reducing bacterium, was also reported to be catalyzing the reduction of perchlroate, chlorate and several other electron

acceptors such as nitrate, iodate and bromate. In other chlorate reducing bacteria, that are unable to reduce perchlorate, unique chlorate reductases have been found to exist (Kengen, Rikken, Hagen, van Ginkel, & Stams, 1999).

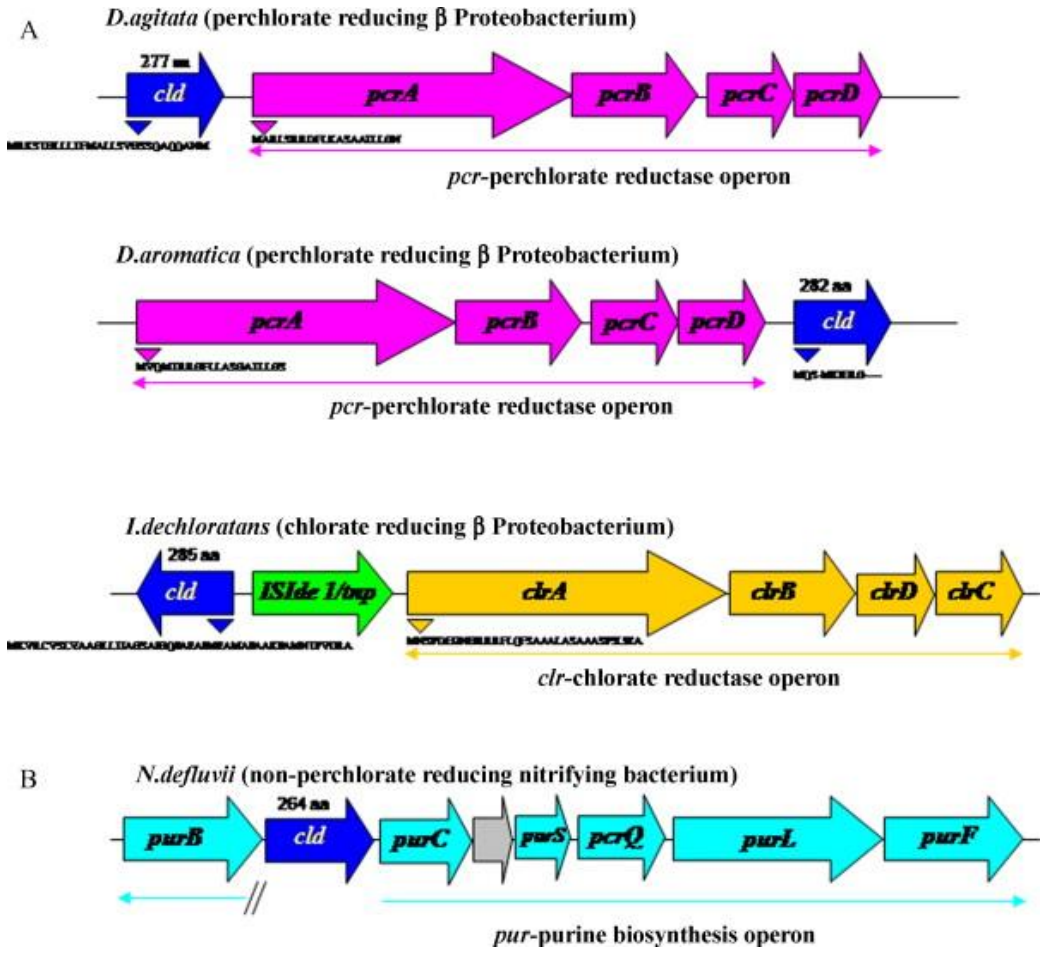

Figure 1.2 : (A) Comparison of gene cluster of perchlorate and chlorate reduction. Number of amino acid residues are indicated for cld gene product. Arrows indicate location of genes and direction of transcription. Cld - chlorite dismutase gene; pcr (A-D) - perchlorate reductase operon; clr (A-D) - chlorate reductase operon; ISIde1/tnp - insertion sequence/transposase gene. (B) Gene cluster of chlorite dismutase in non-perchlorate reducer nitrifier "*Candidatus Nitrospira defluvii*". Cld - chlorite dismutase gene; pur - purine biosynthesis genes (Reprinted from Bardiya and Bae (2011)).

Chlorite dismutase is found in the outer membrane of all perchlorate reducing bacteria (Bruce, Achenbach, & Coates, 1999). This enzyme is a universallly conserved heme-containing homotetramer enzyme (Goblirsch, Streit, Dubois, & Wilmot, 2010; A. Q. Lee, Streit, Zdilla, Abu-Omar, & DuBois, 2008). The chlorite is degraded into oxygen and chloride by a heme b-dependent, non-energy yielding mechanism (Bender, O'Connor, Chakraborty, Coates, & Achenbach, 2002). The chl*orite dismutase has been purified and characterized only from six strains, i.e.* A.

Oryzae GR-1, *D.agitata, I.dechloratans, D. Aromatica* RCB, *P. chloritidismutans* and *N. defluvii* (Kostan et al., 2010). The comparison of gene cluster of perchlorate reduction and chlorate reduction and gene cluster of chlorite dismutase in non-perchlorate reducer nitrifiers are shown in Figure 1.2.

Over all, the purified chlorite dismutases from the perchlorate reducing bacteria have been found to be similar in terms of molecular mass, specific activity and structure. However some differences have been observed in the subcellular localization, genomic organization and transcriptional direction of the cld gene (Bender, Rice, Fugate, Coates, & Achenbach, 2004). In *I. dechloratans*, *D. aromatica* and *D. agitata* chlorite dismutase is encoded by cld ORF and cld gene and perchlorate reductase genes (pcr) are found in close proximity (Figure 1.2-A) (Bender et al., 2005). On the contrary, in *N.defluvii*, the cld gene has been found to be part of a gene cluster (Figure 1.2-B) which contains six genes that are not involved in perchlorate reduction. Chroite dismutase has also been reported from other non-perchlorate reducing bacteria, cyanobacteria and members of archaea such as *Thermus termophilus*, *Haloferax volcanii* and *Magnetospirillum magnetotacticum*(Bardiya & Bae, 2011).

1.2.1 Heterotrophic Reduction of Nitrate and Perchlorate

There are many approaches for the biological reduction of perchlorate in the laboratory and at a large scale (Hatzinger, 2005). In general, one of several different organic substrates is added to a perchlorate-containing soil or groundwater. These substrates are then utilized by indigenous perchlorate-degrading bacteria as an electron donor in the reduction of the perchlorate molecule, with perchlorate serving as the terminal electron acceptor in a form of anaerobic respiration. However, these strains are facultative anaerobes, capable of utilizing oxygen, nitrate and perchlorate as terminal electron acceptors (Coates & Achenbach, 2004).

In the reduction of perchlorate, acetate, sucrose, glucose ethanol and yeast extract could be used as electron donors. The use of acetate as an electron source in the reduction of perchlorate can be illustrated by the following reaction (Coates & Jackson, 2009):

$$CH_3COOH + ClO_4^- \rightarrow 2CO_2 + Cl^- + 2H_2O \qquad (1.2)$$

Nor et al. (2011) studied perchlorate reduction to treat wastewater containing high concentrations of perchlorate (1200 mg/L) with acetate as the electron source. Increasing acetate concentrations resulted in increasing perchlorate reduction rates. However, after a certain acetate concentration, reduction rates remained the same; 1.6 g/L acetate concentration found to be the optimum rate for 1500 mg/L initial perchlorate concentration (Nor et al., 2011).

When an excess of acetate was supplied over the optimal ratio, the perchlorate reduction rate increased linearly with increasing acetate concentration, and the maximum perchlorate reduction rate was obtained at acetate to perchlorate ratio of 3.62 mg-COD/mg-perchlorate (4.0 g-acetate/L). Additional amounts of acetate over this ratio provided no further benefit to perchlorate reduction kinetics (Nor et al., 2011). It is desirable to avoid adding excessive amounts of electron donor to higher levels than the optimal COD/perchlorate ratio since the excess supply of the electron donor increases operating cost. Additionally, the accumulation of the residual electron donor can stimulate the growth of sulfate reducing bacteria, which can produce sulfides and inhibit the activity of perchlorate reducing cultures.

Among electron donors, acetate provided the highest perchlorate reduction followed by ethanol, yeast extract, sucrose, and glucose, indicating that acetate is the most suitable electron donor for perchlorate reduction (Nor et al., 2011).

Butler et al, (2010) studied the reduction of perchlorate in a two chamber microbial fuel cell with denitrifying biocathode. During their study, they observed that 24 mg/(L.d) perchlorate reduction was achieved with electrons from acetate. The acetate they used was in a concentration of 200 mg/L and the authors noted that acetate might cross over from the anode when cathodic conversion efficiency was not 100%. In addition, they found that during early periods of reactor operation, organic carbon originating in the inoculum might be washed out of the system. These findings suggests that there may be organic substrates in the effluent (Butler, Clauwaert, Green, Verstraete, & Nerenberg, 2010).

Wasik et al investigated methanol-based heterotrophic denitrification coupled with microfiltration in a packed bed reactor. The denitrification rate was 10.2 kg NO_3^-

/m^3.day with a HRT of 0.2 h. Effluent nitrate concentrations were below the permissible value of 44 mg NO_3^-/L (Wasik, Bohdziewicz, & Błaszczyk, 2001).

One of the main disadvantages of heterotrophic reduction is the cost of electron donor. In laboratory scale applications, a wide range of donors could be used, including methanol, ethanol, glucose and sucrose. However, in full-scale applications, donor cost must be considered. In a study where two anaerobic CSTRs were used to reduce perchlorate, cheese whey and yeast extract mixture were used as electron donors. However, authors noted that when these donors were replaced with desugared molasses due to cost related problems, efficiency improved (Hatzinger, 2005).

When organic electron donor is utilized to reduce perchlorate, it is crucial to adjust the quantity of organic donor properly. Excess organic donor addition (Excess COD), will result in the residual – not oxidized, organic matter in the effluent, a situation that is completely unwanted in drinking water treatment. It is therefore questionable whether it is acceptable of economically feasible to supply the necessary electrons by adding organic substrates groundwater and drinking water.

Similarly, to perchlorate reduction, heterotrophic denitrifiers utilize simple organic compounds such as carbon and an electron source. High denitrification is the main advantage of the process. However, nitrite may accumulate in the system when substrate is stoichiometrically insufficient. In addition, when excessive substrate is added, it may be present in the effluent (Sierra-Alvarez et al., 2007). In practice, the addition of exactly the right amount of organic substrate is difficult to achieve (Oh, Yoo, Young, & Kim, 2001). Although the utilization of organic substrates has several risks, many researchers have reported successful nitrate reduction using organic substrates.

Denitrification is a well-known process and many studies have reported on electron donors in denitrification processes. Dalmacija et al. (1991) used ethanol as the source of carbon and used denitrifying bacteria to remove nitrate from river water that contained about 117 mg NO_3^-/L. A high efficiency of NO_3^- removal was achieved (~100%). In another study, sugar or glucose syrup was used as substrate and the denitrification rate was higher than 80%, while the influent NO_3^- concentration was 400 mg/L (Nurizzo & Mezzanotte, 1992).

In denitrification process, several types of organic compounds have been used as substrates but acetic acid, ethanol, and methanol are the most commonly reported electron donors for denitrification (Gayle, Boardman, Sherrard, & Benoit, 1989).

1.2.2 Autotrophic Reduction of Nitrate and Perchlorate

Microorganisms carry out an autotrophic reduction using inorganic compounds as electron donors, including: hydrogen and reduced iron or sulfur compounds (Sahu et al., 2009). Various designs of hydrogen-based reactors have been tested in laboratory studies on nitrate (K. C. Lee & Rittmann, 2002) chlorate (Kroon & van Ginkel, 2004) and perchlorate treatment in drinking water (Logan & LaPoint, 2002; Robert Nerenberg, Rittmann, & Najm, 2002). The expected advantage of these systems is the absence of any remaining organic electron donor in the treated water. Additionally, some of the inorganic electron donors are extremely cheap compared to the organic electron donors (i.e. elemental sulfur).

Urbansky and Schock (1999) studied hydrogen utilizing an autotrophic microbial consortium capable of reducing nitrate and perchlorate simultaneously in batch culture. Reduction to below the detection limit of 5 and 0.005 mg/L, respectively was achieved in 48 hours.

Using hydrogen to maintain autotrophs resulted in high reduction rates. However a limiting factor may be the availability of hydrogen for the organisms. Hydrogen delivered to organisms by simply bubbling the reactor was reported to be insufficient for biomas maintainance. Another drawback has been difficulty with pH adjustment. To solve this problem, bicarbonate has been supplied to the system, but this led to an increase in the total operational cost (Urbansky & Schock, 1999).

Another hydrogen-based study was done by Nerenberg et al. (2006). They determined the kinetic parameters for a hydrogen-oxidizing perchlorate-reducing bacteria and found that, oxygen and nitrate were also required to reduce perchlorate at low concentrations (<14 μg/L) (Robert Nerenberg, Kawagoshi, & Rittmann, 2006). In another study, hydrogen gas (5%) along with carbon dioxide, was provided to achieve 30 – 39 % perchlorate reduction in an autotrophic bioreactor (Logan & LaPoint, 2002).

Despite of the fact that hydrogen exhibits good reduction efficiency, it has a number of drawbacks. Hydrogen is relatively expensive and dangerous to handle because of its explosive nature (Son, Lee, Chiu, Kim, & Cha, 2006).

Fe^0, is inexpensive, safe to handle and does not leave organic residue in the treated water. It is a strong reducing agent (E^0 = -0.44V), and has been used in recent years to treat oxidized pollutants such as nitroaromatics, nitramines, and azo dyes through reductive transformation (Perey, Chiu, Huang, & Cha, 2002). Thermodinamically, perchlorate is readily reducible by Fe^0. However, studies have shown that this reaction is very slow under ambient conditions, suggesting that the energy barrier to the reaction is large (Moore, De Leon, & Young, 2003). Under anaerobic conditions, iron corrosion produces hydrogen gas through the reduction of protons. In the presence of hydrogenotrophic microorganisms, cathodic hydrogen may be utilized to degrade perchlorate.

Shrout et al. (2005) studied microbial perchlorate reduction in the presence of zero valent iron using a mixed culture obtained from an anaerobic digester. However, they reported that the addition of Fe^0 to the anaerobic culture resulted in a slower rate of perchlorate reduction. A similar study done by Yu et al. (2006). They showed that zero valent iron was capable of serving as an electron donor for perchlorate reduction by providing hydrogen to hydrogen utilizing autotrophs. Similarly, Son et al. (2006), also used zero valent iron as an electron donor in both batch and column reactors. In the batch tests, 65 mg/L perchlorate was reduced in 8 days, demonstrating the potential applicability of zero valent iron as a source of electrons for biological perchlorate removal (Son et al., 2006).

Ju et al., (2007) enriched a S^0 oxidizing ClO_4^- reducing consortium from a wastewater seed. This consortium reduced ClO_4^- at a rate of approximately 2.0 g./m^3.h. The presence of NO_3^- delayed the onset of the ClO_4^- reduction, but supplementing yeast extract increased ClO_4^- rates. In more recent research by the same authors (Ju et al., 2008), ClO_4^- was stoichiometrically converted to CI^- using S^0 as an electron donor in batch cultures seeded with activated sludge.

Inorganic electron donors such as reduced sulfur compounds (Furumai, Tagui, & Fujita, 1996) and elemental sulfur (Ahmed et al., 2012; Sahinkaya & Dursun, 2014; Sahinkaya, Hasar, Kaksonen, & Rittmann, 2011; Sahinkaya, Kilic, Altun, Komnitsas,

& Lens, 2012; Sahinkaya, Kilic, Calimlioglu, & Toker, 2013; Sengupta, Ergas, & Lopez-Luna, 2007) are also utilized in autotrophic denitrification studies. In a study by Sierra-Alvarez et al. (2007), sulfur-based autotrophic denitrification of groundwater was investigated using a sulfur–limestone packed bed bioreactor. They observed the nearly complete removal of nitrate (7.3 mM) at a loading rate of 21.6 mmol/(L.d) (or 0.3 g NO_3^--N/(L d.)). Batch studies were conducted to evaluate the process kinetics. These studies showed that the denitrification rate is dependent on the surface area of elemental sulfur (Sierra-Alvarez et al., 2007).

In a recent study, Sahinkaya et al. (2015) investigated nitrate reduction by elemental sulfur in a membrane bioreactor. Sulfur was added to the reactor according to theoretical requirement. Almost complete nitrate reduction was observed when the influent nitrate concentrations were 25-50 mg NO_3^--N/L with a HRT as low as 5h (Sahinkaya, Yurtsever, Aktaş, Ucar, & Wang, 2015).

In a study conducted by Lee and Rittmann, a H_2 based hallow fiber membrane reactor was used to (1) partially remove nitrate, (2) minimize hydrogen wasting and (3) accumulate low nitrite. The system could achieve partial nitrate removal up to 92%. The influent NO_3^--N was 15 mg/L, but the effluent was decreased to 0.2 mg N/L (K. C. Lee & Rittmann, 2002).

In conclusion, both heterotrophic and autotrophic processes have been used to reduce nitrate and perchlorate (Bardiya & Bae, 2011; Matějů, Čižinská, Krejčí, & Janoch, 1992; E. J. McAdam & Judd, 2006; Srinivasan & Sorial, 2009). When planning of treatment plants, the advantages and drawbacks of both processes should be considered carefully. The parameters that must be taken are : the risk of residual electron donor presence in the effluent, the risk of end product presence in the effluent, the electron donor process kinetics, by-products produced during the process, and efficiency

1.3 Unique Aspect

It is estimated that the majority of water sources that are polluted with perchlorate have not been discovered and are currently treated as safe water. A new perchlorate detection method was developed in 1997, leading to perchlorate detection at concentrations as low as 4 μg/L (Hautman, Munch, Eaton, & Haghani, 1999). Then,

measurement sensitivities increased to detect perchlorate concentrations as low as 55 ng/L using two-dimensional IC with suppressed conductivity (Wagner et al., 2007). Since the development of this technology, perchlorate has been detected in foods such as water, fish, rice and milk (Srinivasan & Sorial, 2009; A. Sungur & Sangün, 2011; Ş. Sungur & Atan, 2012).

Perchlorate in drinking water may contribute to a number of health and wellness issues, such as tiredness. More seriously, it is possible that perchlorate may play a role in a wide array of metabolic diseases, such as heart failure, a slowdown in heart rhythm, constipation and apnea (short time respiratory arrest) could be the undiscovered presence of perchlorate in the drinking water. In a study conducted by Chen et al. (2013), two groups of workers, that differed on their perchlorate exposure (one exposed, the other not) were compared. Serious systolic blood pressure rise was observed in the workers who had been exposed to perchlorate. Significant decrease in thyroid function was also observed in the exposed group (Chen, Shao, Wu, Li, & Peng, 2013). In a study conducted with healthy adult volunteers in China, it was determined that perchlorate levels above 0.007 milligrams per kilogram per day (mg/(kg·d)), could temporarily inhibit the iodine absorption by the thyroid gland (Greer, Goodman, Pleus, & Greer, 2002). The EPA calculated a reference dose by dividing 0.007 by the standard intraspecies uncertainty factor of 10. The agency then assumed a person's weight is 70 kg, and consumes 2 liters of drinking water per day and calculated a drinking water equivalent level of 24.5 µg/L (0.0007 mg/(kg.d)*70 kg/2 L = 24.5 µg/L (Tikkanen, 2006). The only study in Turkey on groundwater perchlorate levels was conducted in Hatay. This study found that perchlorate concentrations were relatively low and concluded that they do not pose a risk for human life (Ş. Sungur & Atan, 2012). However, the cumulative effect of even low concentrations of perchlorate is unknown. With this in mind, perchlorate concentrations were assessed in 22 wells in the Harran Plain of Turkey. We did not detect any perchlorate. This study was the first to search for perchlorate in the groundwater from the Harran Plain, which is highly polluted with nitrate. Nitrate concentrations were between 5.93 and 83 mg NO_3^--N/L.

Nitrate, the most common groundwater contaminant, is highly regulated and measured internationally. Researchers have used many methodologies for the treatment of these pollutants, but the biological treatment is the most efficient

method since it does not produce a concentrated brine (reverse osmosis – nanofiltration) or a sludge (adsorption) to deal with. Although microorganisms reduce the perchlorate into harmless Cl^- and O_2, the drawbacks mainly originate from the substrate. The presence of organic residuals in the effluent of heterotrophic processes is concerning. This risk is lower in autotrophic processes, but the slow kinetics of the process and sensitivities of the microorganisms also pose a drawback. Therefore, it is necessary to establish a combined autotrophic and heterotrophic processes when treating high concentrations of nitrate and perchlorate. The advantages of this combination include effluent sulfate, acidity and low risk of organic effluent. Another novel aspect of the thesis was the establishment of the appropriate combination of autotrophic and heterotrophic processes. Therefore, one mixotrophic single reactor and one mixotrophic sequential process (heterotrophic-autotrophic) was operated. The obtained data was compared with other autotrophic and heterotrophic reactors that treat nitrate and perchlorate.

1.4 Organization of the Thesis

In this thesis, 4 continuous up flow column, 1 sequential system and several batch reactors were operated. At first, 2 autotrophic and 1 heterotrophic reactor was established and operated for more than 100 days. Independently from these reactors 1 mixotrophic reactor was established and operated for 173 days and then heterotrophic autotrophic sequential system was established and operated for 100 days. The sequential system was then utilized for real groundwater treatment polluted with 74 mg NO_3^--N/L.

The operational conditions and the performance data for each reactor was presented in related chapter in this thesis. Table 1.5 below presents the operational informations and the locations of such data in the thesis. Table 1.6 also presents the publication informations for each reactor. General operational conditions are presented below.

General specifications of the reactors:

- The reactors were glass columns with 400 ml empty bed volume.
- The reactor was filled with either elemental sulfur alone (0.5–1 mm), or the mixture of elemental sulfur and limestone (0.5– 1 mm) or sand (0.5–1 mm).

- All reactors were covered with aluminum foil.
- Up flow feeding with adjustable peristaltic pumps
- Temperature was 28–30 °C in a temperature controlled room.

Table 1.6 : Basic informations of the reactors and publication-chapter informations.

Reactor Name	**Carrier Material**	**Feed Information**	**Chapter**	**Publication**
$NaHCO_3$ fed autotrophic	Elemental sulfur	NO_3^--N: 25 mg/L HRT: 12 to 2 h. NO_3^--N load: 50 – 300 mg N/(L.d) ClO_4^-: 50 – 1000 µg/L $NaHCO_3$: 375 mg/L	III	DOI: 10.2166/ws.2015.129
Limestone fed autotrophic	Elemental sulfur and limestone	NO_3^--N: 25 mg/L HRT: 12 - 2 h. NO_3^--N load: 50 – 300 mg N/(L.d) ClO_4^-: 50 – 1000 µg/L	II	DOI: 10.1002/jctb.4744
Methanol based heterotrophic	Sand	NO_3^--N: 25 mg/L HRT: 12 to 1 h. NO_3^--N load: 50 – 600 mg N/(L.d) ClO_4^-: 50 – 1000 µg/L	II	DOI: 10.1002/jctb.4744
Mixotrophic	Elemental sulfur and limestone	NO_3^--N: 25 mg/L Methanol: 25 – 35 mg/L HRT: 12 – 1.5 h. NO_3^--N load: 50 – 400 mg N/(L.d) ClO_4^-: 50 - 1000 µg/L	III	DOI: 10.2166/ws.2015.129
Sequential system	Sand and elemental sulfur	NO_3^--N: 74 mg/L Methanol: 170 mg/L HRT: 2 h. NO_3^--N load: 888 mg N/(L.d) ClO_4^-: 500 – 1500 µg/L	IV	DOI: 10.1080/09593330.2015.1065009

Feed informations of the reactors

- The feed was contained different concentrations of KNO_3, K_2HPO_4, $NaClO_4*H_2O$, $NaHCO_3$ and C_2H_5OH,
- The feed was analyzed for NO_3^--N, ClO_4^-, Cl^-, NO_2^--N, DOC, pH, and alkalinity
- Feed was prepared with tap water and doxygenated by passing through N_2 gas for 20 min.
- The feed was then kept under anaerobic conditions in collapsible feed containers.

Finally, effluent was analyzed for NO_3^--N, NO_2^--N, ClO_4^-, ClO_3^-, ClO_2^-, Cl^-, SO_4^{2-}, HS^-, (DOC), pH and alkalinity. The analytical methods were presented in Table 1.7 below.

Table 1.7 : The analytical methods used in the thesis.

Analysis	Method	Reference
Chlorate, chlorite, chloride, nitrate, nitrite, and sulfate	EPA Method 300.1 and 317.0 DIONEX AS9 column with suppressed conductivity detector. Chromatographic conditions: • Flow rate: 1 ml/dk • Oven temp: 35 °C • Injection volume : 25 µL • Eluent : 9 mM $NaHCO_3$	(Thermo Scientific 2013)
Perchlorate	EPA Method 314.1 (for confirmation) DIONEX AS9 column with suppressed conductivity detector. Chromatographic conditions: • Flow rate: 0.25 ml/dk • Oven temp: 35 °C • Injection volume : 100 µL • Eluent : 35 mM NaOH	(Thermo Scientific 2012)
Dissolved organic carbon	EPA Method 415.1 TOC determination by combustion	(Visco, Campanella, & Nobili, 2005)
Alkalinity	Standard Methods for the Examination of Water and Wastewater, Method No : 2320	(Rice, Baird, Eaton, & Clesceri, 2005)
Sulfide	Spectrophotometrical measurement at 480 nm decribed by Cord-ruwisch, 1985	(Cord-ruwisch, 1985)
Microbial Community Analysis - 1	Real time PCR, with Fast DNA spin and MS condenser	(Derveaux et al., 2010)
Microbial Community Analysis - 1	PCR-DGGE method, with universal primers.	-
Chemical Oxygen Demand	Standard Methods for the Examination of Water and Wastewater, Method No : 5220	(Rice et al., 2005)

The thesis contains three data chapters (Chapters 2-5) following the introduction chapter (Chapter 1). Lastly, a conclusions and recommendations chapter (Chapter 6) is provided. Each data chapter contains the comparative results of the operated reactors except Chapter 4 in which the performance of a sequential system was provided. Specifically, the simultaneous nitrate and perchlorate reduction efficiencies and by-products of the each individual process were presented. In total, six reactors were operated at least 100 days. Autotrophic and heterotrophic reactors used elemental sulfur and methanol as the electron donor, respectively. In the mixotrophic reactor, methanol (less than the stoichiometric requirement to reduce all influent nitrate and perchlorate) was added to the feed of autotrophic reactor and heterotrophic denitrification was provided in the reactor makes the whole system mixotrophic.

Chapter 2 presents data on the comparison of an autotrophic and heterotrophic reactor. The autotrophic reactor was filled with elemental sulfur and limestone mixture and the reactors were operated for 122 days. Nitrate load, for both reactors, was 50 mg N/L.d in the first period but then increased to 300 and 600 mg N/L.d for autotrophic and heterotrophic reactors respectively. Although perchlorate reduction was inhibited by nitrate reduction, 95% perchlorate removal was observed when initial perchlorate was 1000 µg/L. Effluent DOC concentrations were between 2.4 – 4 mg/L and on some occasions, it was increased up to 7.5 mg/L.

The data on the comparison of the mixotrophic reactor and autotrophic reactor was presented in Chapter 3. The autotrophic and mixotrophic reactors were operated for 369 and 174 days, respectively. More than 97% perchlorate removal was observed in a period when influent was 1000 µg/L. Average effluent sulfate concentration was 259± 87.70 mg/L for the autotrophic reactor. However, for the mixotrophic reactor, effluent sulfate concentrations were 176.10± 11.50 mg/L and 150.25± 16 mg/L when 25 and 35 mg/L methanol was added. Effluent DOC concentration of the mixotrophic reactor was below 2 mg/L.

Chapter 4 presents the data on the sequential process in which effluent of heterotrophic reactor was pumped to autotrophic reactor influent. The system was operated for 100 days and nitrate concentration was increased from 25 to 100 mg/L throughout the study. Perchlorate concentration was 1000 µg/l for all periods and at the end of the autotrophic reactor concentrations of both anions were below detection limits. Although influent nitrate was increased to 100 mg/L, effluent sulfate concentrations were below 250 mg/L except few days because of the addition of methanol to heterotrophic reactor. Methanol addition was performed to reduce a certain portion of influent nitrate nitrogen leaving a stable concentration of nitrate nitrogen in the autotrophic reactor influent (Influent NO_3^--N concentration – 25 mg NO_3^--N/L was considered to be reduced in heterotrophic reactor). Hence, average effluent sulfate concentrations for the first, second and third periods were 210±37, 200±40.20 and 193±42 mg SO_4^{2-}/L respectively. Although DOC was observed in the effluent of the heterotrophic reactor, effluent DOC concentrations of the autotrophic reactor were below 0.20 mg/L for each period.

Chapter 5 presents data on the batch reactors representing the autotrophic and heterotrophic reactors. In this chapter, two elemental sulfur-based autotrophic

processes with different alkalinity sources ($NaHCO_3$ and limestone fed) and a methanol-based heterotrophic process were investigated to achieve simultaneous nitrate and perchlorate reduction in ground water. In batch reactors, excellent nitrate reduction with $NaHCO_3$-fed autotrophic and heterotrophic processes was obtained under 25 mg/L NO_3^--N and various (100–1500 μg/L) initial perchlorate concentrations. Average nitrate reduction for the limestone-fed reactor was around 95% by the end of 90 hours. Complete perchlorate reduction was observed in 72 hours for the heterotrophic reactor whereas the $NaHCO_3$-fed autotrophic reactor showed 97% perchlorate removal by the end of 90 hours. The perchlorate reduction performance of the limestone-fed reactor varied between 51.8% and 92% depending on the initial perchlorate concentration. Sulfate was produced as a result of elemental sulfur-based autotrophic denitrification: 257.20±12.50 and 238.90±28.9 mg/L SO_4^{2-} were produced for the $NaHCO_3$ and limestone fed reactors respectively.

Finally, chapter 6 presents this thesis' conclusions and recommendations for future research.

2. SIMULTANEOUS NITRATE AND PERCHLORATE REDUCTION USING SULFUR-BASED AUTOTROPHIC AND HETEROTROPHIC DENITRIFYING PROCESSES

2.1 Introduction

Perchlorate (ClO_4^-) is a salt derived from perchloric acid and may be released into the environment, from either natural or anthropogenic sources. Anthropogenic sources include defense and aerospace industries, as well as fireworks and road flares (Okeke, Giblin, & Frankenberger, 2002). Ammonium perchlorate is used as an oxidant in propellants for missiles and rockets. Pharmaceutical industries use perchlorate in the form of $KClO_4$ to treat hyperthyroidism (Srinivasan & Sorial, 2009). Perchlorate may reach groundwater via some natural processes (Orris et al., 2003). Perchlorate blocks iodine uptake by the thyroid, decreasing the thyroid hormone (triiodothyronine-T3 and thyroxine-T4) concentrations (Greer et al., 2002). Therefore, perchlorate can trigger many diseases related to the basal metabolism.

Prior to 1997, the minimum detection limit for perchlorate with gravimetric liquid–liquid extraction and spectrophotometry was 100 μg/L. However, the development of an ion chromatographic method with suppressed conductivity allowed perchlorate detection limits to 4 μg/L. Perchlorate was added to the drinking water Contaminants Candidate List in March 1998, and some states in the USA established advisory levels for perchlorate such as California (6 μg/L), Massachusetts (2 μg/L) and Texas (4 μg/L) (Srinivasan & Sorial, 2009).

Perchlorate is commonly present in groundwater along with nitrate (McCarty & Meyer, 2005), and nitrate may cause methemoglobinemia in infants, malformation and mutation when transformed into nitrosoamines (Della Rocca et al., 2007). In the

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USA, nitrate concentrations in the 10–25% of total groundwater used as drinking water are above 10 mg NO_3^--N/L (Liu, Jiang, Wan, & Qu, 2009).

In addition, some oxidized compounds such as nitrate, nitrite, chlorate and phosphate are found in the effluents of several industries (Kengen et al., 1999). Because of the fast reaction rate, the most common method for detoxification of these oxyanions is biological reduction, eliminating the need for expensive catalysts or chemicals. Biological reduction of nitrate and ClO_4^- can be achieved using organic (Sahinkaya & Dursun, 2012; Wang, Liu, & Qin, 2012), or inorganic electron sources (Demirel, Uyanık, Yurtsever, Çelikten, & Uçar, 2014; Ju et al., 2007; Sahinkaya et al., 2015). For heterotrophic processes, acetate, lactate, pyruvate, casamino acids, fumarate, succinate, methanol and ethanol are the common carbon and electron sources (Bardiya & Bae, 2011). Reduction of nitrate in the presence of methanol is shown in Equation (2.1). The main disadvantage of the process is the difficulty of dosing proper concentrations of organic substrate. If carbon is added at more than the stoichiometric requirement, effluent may be contaminated with unused organic, in the contrary case nitrite may accumulate, which is more toxic than nitrate.

$$NO_3^- + 1.08\ CH_3OH + 0.24H_2CO_3 \rightarrow 0.056\ C_5H_7N_2O + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (2.1)$$

Alternatively, inorganic electron donors such as hydrogen, zero valent iron, and elemental sulfur may also be used for the bioreduction of oxyanions, especially for drinking water treatment (Bardiya & Bae, 2011). In sulfur-based process, elemental sulfur and nitrate/perchlorate act as respective electron donors and acceptors, without requiring organic supplementation (Equation 2.2). As elemental sulfur is non-toxic, water insoluble, stable under normal conditions, and readily available, much more attention has been paid to sulfur-based autotrophic denitrification of nitrate-contaminated groundwater (Sahinkaya, Dursun, et al., 2011; Soares, 2002). However, this process has the disadvantage of generating sulfate and acidity (Reaction 2). Therefore, external alkalinity supplementation is required to keep the pH neutral during the process. Limestone is generally used to provide alkalinity for the system (Ju et al., 2007).

$$1.1S^{0}+NO_3^-+0.76H_2O+0.4CO_2+0.08NH_4^+ \rightarrow 0.08C_5H_7O_2N+1.1SO_4^{2-}+0.5N_2+1.28H^+ \quad (2.2)$$

Sulfur-based autotrophic denitrification can also be used for simultaneous nitrate and chromate reduction (Sahinkaya & Kilic, 2014) Therefore, this process can alternatively be used for simultaneous reduction of nitrate and other oxidized contaminants, like perchlorate. Generally perchlorate concentrations in groundwater sources are too low (<1 mg/L) to sustain bacterial growth when perchlorate is the sole electron acceptor. Hence, nitrate may be required as the primary electron acceptor. However, high concentrations of nitrate may also inhibit perchlorate reduction (London, De Long, Strahota, Katz, & Speitel, 2011). Therefore, continuous lab scale studies are needed to understand the process performance under varying operational conditions and to get some design criteria before its use in full-scale applications. To the best of our knowledge, this is the first study in the literature about comparative performance assessment of sulfur-based autotrophic and heterotrophic denitrification processes for simultaneous perchlorate and nitrate reduction.

2.2 Materials and Methods

2.2.1 Denitrifying Column Bioreactors

Two denitrifying up-flow column bioreactors were operated in parallel. Elemental sulfur and methanol were used as electron sources in Reactor 1 and Reactor 2, respectively. The working volume of the glass reactors (R1 and R2) was 500 mL. Reactor 1 was packed with the mixture of elemental sulfur granules (0.5–1 mm) and limestone (1–2 mm), whereas reactor 2 was packed with sand particles (0.5–1 mm). The volumetric ratio of elemental sulfur to limestone was selected as 2/1, based on the results of a previous study (Kilic, Sahinkaya, & Cinar, 2014). The autotrophic bioreactor (R1) was inoculated with a sludge obtained from an existing denitrifying autotrophic reactor operated at 28–30°C, which is within the optimum range for perchlorate reduction (Ghosh, Pakshirajan, Ghosh, & Sahoo, 2011). The heterotrophic bioreactor (R2) was inoculated from the anoxic compartment of a real-scale Bardenpho process. Oxygen dissolved in the feed containers was stripped using N_2 gas, and the feed containers were kept at 4 °C to prevent possible microbial

growth. The reactors were covered with aluminum foil to prevent any phototrophic growth.

The reactors were fed with tap water supplemented with KNO_3, K_2HPO_4 and ClO_4^-. The feed of R2 was supplemented with methanol as a carbon source. The reactors were sampled at least three times a week for the measurement of NO_3^--N, NO_2^--N, SO_4^{2-}, pH, dissolved organic carbon, alkalinity, HS^-, ClO_2^-, ClO_3^-, ClO_4^- and Cl^-. The influent was also sampled once a week to measure the same parameters except sulfide.

2.2.2 Reactor Operation

Operational conditions of the bioreactors are presented in Table 2.1. During the first period of the study, HRTs were kept at 12 h to enrich denitrifiers, and were gradually decreased and kept at around 2 h and 1 h in autotrophic and heterotrophic reactors, respectively. The feed of the heterotrophic bioreactor was supplemented with methanol at a concentration of 100 mg/L, corresponding to C/N ratio of 1.5. McAdam and Judd (2007) reported optimum C/N ratio ranged from 1.45 to 1.52, at which effluent organic, nitrate and nitrite concentrations were low (Ewan J. McAdam & Judd, 2007). Perchlorate was added to influent at a concentration of 50 μg/L in the 4th period and the concentration was gradually increased to 1000 μg/L in the last period. The column reactors were operated for around 122 days under nine different operational conditions (Table 2.1).

2.2.3 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 (4mm× 250 mm). Perchlorate was measured by DIONEX 500 system (suppressed conductivity) with Ion-Pac AS20 column. Alkalinity and COD were measured according to Standard Methods. Sulfide was measured spectrophotometrically at 480 nm using a Shimadzu UV-VIS spectrophotometer following the method described by Cord-ruwisch (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 ions and sulfide measurements.

Table 2.1 : Operational conditions for both R1 (autotrophic) and R2 (heterotrophic).

Periods	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Days	0-46	47 - 57	58-67	68 – 78	79 – 85	86-96	97-107	108-115	116-122
NO_3^--N (mg/L)	25	25	25	25	25	25	25	25	25
NO_3^--N load$_{Auto}$ (mg N/L day)	50	150	300	300	300	300	300	300	300
NO_3^--N load$_{Hetr}$ (mg N/L day)	50	300	600	600	600	600	600	600	600
ClO_4^- (µg/L)	-	-	-	50	100	200	300	500	1000
HRT$_{Auto}$ (hours)	12	4	2	2	2	2	2	2	2
HRT$_{Hetr}$ (hours)	12	2	1	1	1	1	1	1	1

2.2.4 Batch Adsorption Experiments

Batch adsorption experiments were conducted to identify NO_3^- and ClO_4^- removal mechanisms in the absence of biomass. In the experiments, 100 mL serum bottles were supplemented with 25 mg NO_3^--N/L and 1000 µg ClO_4^-/L. For the autotrophic denitrification experiments, serum bottles were supplemented with 1.5 g elemental sulfur and 1 g limestone. Similarly, for the heterotrophic denitrification experiments, serum bottles were supplemented with 1 g sand and 100 mg CH_3OH/L. All serum bottles were operated in a temperature controlled room at 30 °C and regularly sampled for the measurement of NO_3^--N and perchlorate.

2.3 Results

2.3.1 Perchlorate and Nitrate Removal Performances of the Autotrophic reactor (R1)

Perchlorate (50 µg/L) was fed to the influent of the reactor on day 68 and its concentration was gradually increased to 1000 µg/L at a constant nitrate loading rate of around 0.3 g NO_3^--N/(L.d). In the first period, the nitrate load was 0.05 g NO_3^--N/(L.d) and was increased to 0.15 and 0.3 g NO_3^--N/(L.d) in the period 2 and 3, respectively (Table 2.1). The performance of denitrifying column reactor is presented in Figure 2.1. In the first three periods, effluent nitrate concentration was always below detection limit (0.1 mg/L). When the HRT was decreased to 2 h in period 3, nitrate was detected in the effluent, but its concentration was still <3mg/L. The highest influent perchlorate concentration of 1000 µg/L was in period 9, and the average effluent NO_3^--N concentration in this period was 0.20 mg/L, which indicated denitrification performance was not adversely affected by perchlorate under the

studied conditions. Throughout reactor operation effluent nitrate concentration averaged 0.31 mg NO_3^--N/L.

In the period 4, the influent and effluent perchlorate concentrations averaged 50 μg/L and 22±20.92 μg/L, respectively, corresponding to a 56% reduction. When the influent perchlorate concentration was further increased to 100 μg/L, its effluent concentration averaged 20±19.75 μg/L, corresponding to 80% reduction. When influent perchlorate was further increased to 200, 300, and 500 μg/L in the following periods, the average effluent perchlorate concentrations were 61±30.76, 84±43.53 and 71±76.24 μg/L, respectively (Figure 2.2).

Similarly, influent ClO_4^- concentration of 1000 μg/L was decreased to around 53.36±21.26 μg/L in the effluent, corresponding to a 95% reduction. It is interesting to observe varying effluent perchlorate concentrations independent of influent perchlorate concentrations, which ranged from 50–1000 μg/L. This variation may be due to the slow acclimation of autotrophic bacteria to perchlorate. Although over 95% reduction was attained, the observed effluent perchlorate concentrations may not be acceptable, as some states in the USA have established advisory levels much lower than the values reached by an autotrophic denitrification process; such as 6 μg/L in California, 2 μg/L in Masachusetts, and 4 μg/L in Texas (Srinivasan & Sorial, 2009). Nevertheless, the sulfur-based autotrophic denitrification process may be used as a primary treatment. This process achieved almost complete nitrate removal at 0.3 g NO_3^--N/(L.d) loading, and 95% perchlorate removal at 12 mg/(L.d) loading without external supplementation of organic substrate.

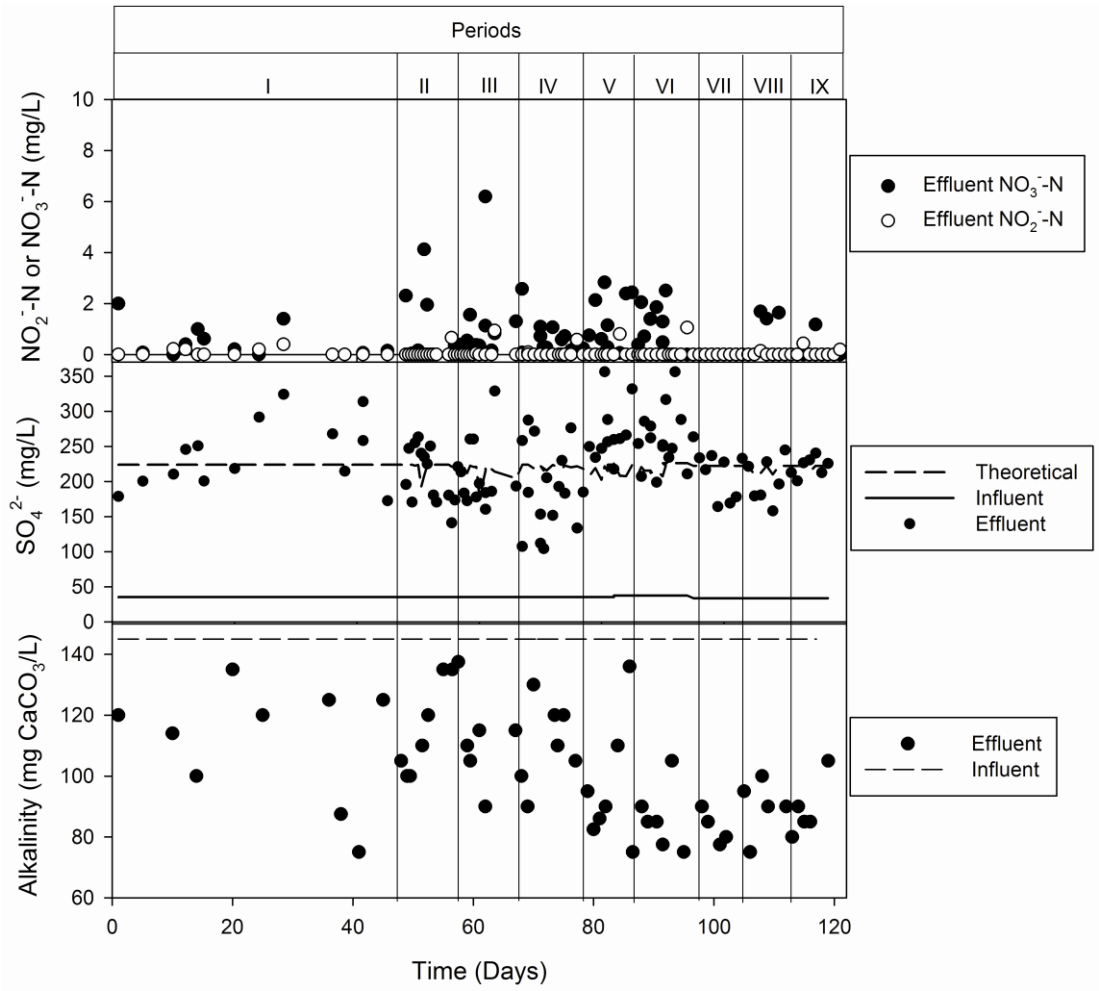

Figure 2.1 : Nitrate, nitrite, sulfate and alkalinity variations of R1. Average influent NO_3^--N concentration was 25±0.20 mg/L and theoretical sulfate concentration was calculated according to Equation 2.2.

Sulfate was produced as a result of the elemental sulfur-based autotrophic denitrification process. Influent sulfate concentration varied between 14 and 43 mg/L with an average value of 31±2.45 mg SO_4^{2-}/L. Theoretically 1 mg NO_3^--N will generate 7.54 mg SO_4^{2-}/L according to Equation 2.2. Theoretically calculated effluent sulfate concentrations are presented in Figure 2.1 together with the measured effluent sulfate concentrations. In the first period (day 0–46), effluent sulfate concentrations were higher than those theoretically calculated. Mean and theoretically calculated sulfate concentrations in this period were 239±16 and 225±14 mg/L, respectively. The fluctuating sulfate concentration may be due to partial oxidation of elemental sulfur with oxygen leaking to the bioreactor from feed and during the operation. In periods 2 and 3, when the HRT was decreased to 4 and 2 h, respectively, effluent sulfate concentrations averaged 233±19 mg/L and 194±16

mg/L. Theoretical and measured sulfate concentrations in periods 4–9 were 218 and 227±30 mg/L.

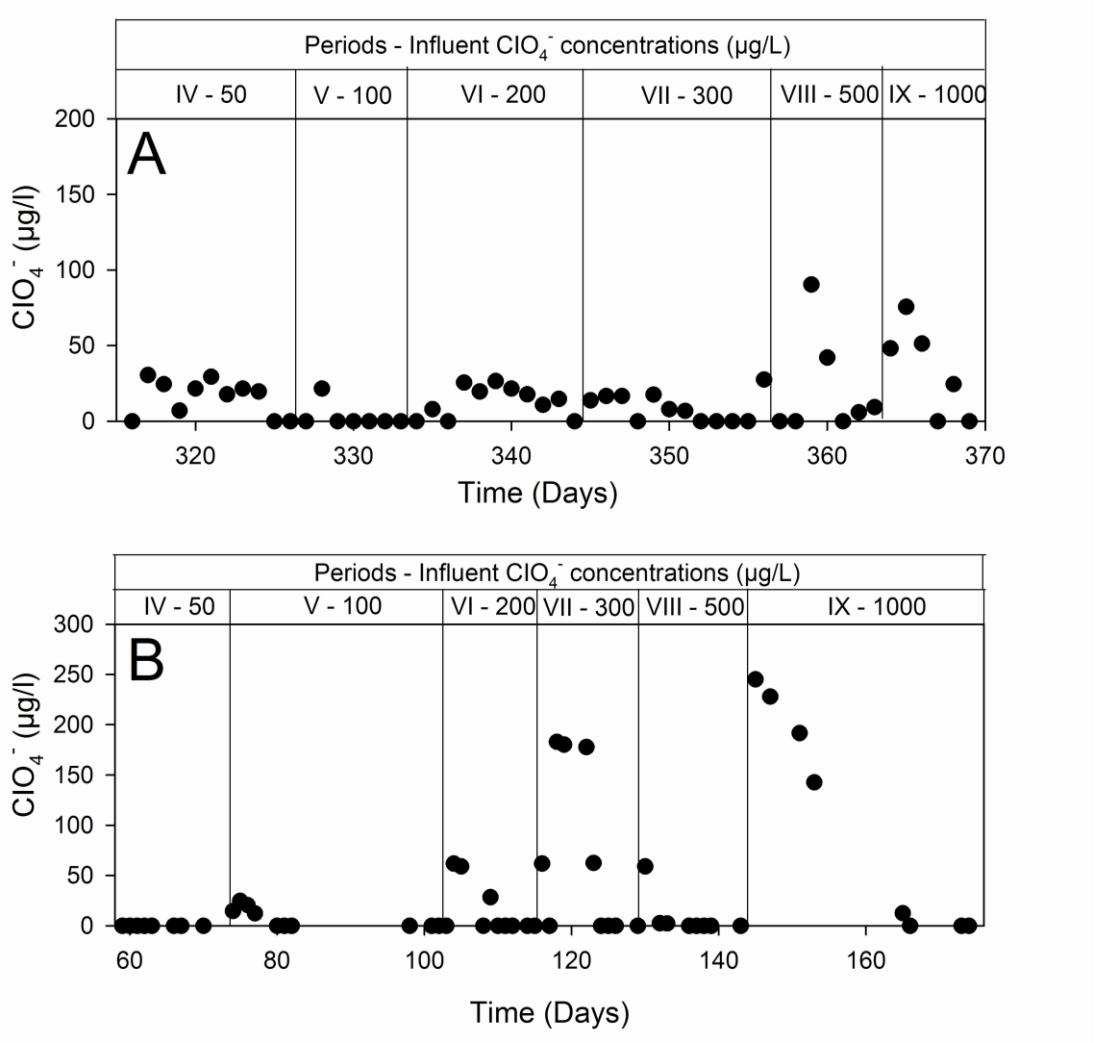

Figure 2.2 : Influent and effluent perchlorate concentrations for R1 and R2.

Theoretically 1 mg NO_3^--N consumes 4.57 mg $CaCO_3$ (Equation 2.2). Based on this stoichiometry, the denitrification of 25 mg NO_3^--N/L consumes 114 mg $CaCO_3$/L. The influent concentration averaged 155±23 mg $CaCO_3$/L. Since the reactor was filled with limestone as a source of alkalinity, effluent alkalinity averaged 96±30 mg/L. This alkalinity was higher than the required minimum concentration of 80 mg $CaCO_3$/L (Metcalf & Eddy, 2003) to keep the pH at neutral (Figure 2.1). The reactor was partially packed with limestone to compensate acid generated during autotrophic denitrification. In the case of limestone absence, complete reduction of 25 mg NO_3^--N/L would require 114 mg $CaCO_3$ and the alkalinity in the bioreactor would decrease as low as 34±5 mg/L, which is not enough to keep the pH neutral. In the autotrophic

bioreactor, average effluent alkalinity was 96±30 mg $CaCO_3$/L because of the alkalinity supplied by the limestone dissolution.

The average influent and effluent pHs were 8.12±0.48 and 7.41±0.28, respectively. Limestone is a readily available material, but because of its low solubility and increasing effluent hardness it may not be preferred in conventional treatment processes. Hence, dissolved alkalinity sources such as $NaHCO_3$ are needed to meet the alkalinity requirements (Sahinkaya & Dursun, 2012).

2.3.2 Performance of Heterotrophic Reactor Fed With Methanol (R2)

Reactor 2 was operated similarly to reactor 1, and the HRT was 12 h in the first period and decreased to 2 h and 1 h in periods 2 and 3, respectively (Table 2.1). In the rest of the operation, the HRT and nitrate loading rates were 1 h and 0.6 g NO_3^--N/(L.d), respectively. Throughout the reactor operation, the effluent nitrate concentration was lower than 0.5 mg NO_3^--N/L, and the nitrate removal performance was not adversely affected by increasing perchlorate concentrations. The heterotrophic reactor outperformed the autotrophic one. Although influent perchlorate concentration was increased gradually to 1000 μg/L, effluent concentrations were below detection limits, except on days 74, 80 and 102, when operational problems were encountered. The effluent sulfide concentration was <1mg/L throughout the study.

Alkalinity concentrations in the effluent of the bioreactor increased, according to Equation 2.1. The theoretical effluent alkalinity concentration was calculated (Figure 2.3) assuming that 3.57 mg $CaCO_3$ would be generated per mg NO_3^--N reduced. The theoretically calculated and measured alkalinity concentrations were in good agreement (Figure 2.3). Although the averages of the measured and theoretical alkalinities were quite similar (244±17 and 233±2.86 mg $CaCO_3$/L, respectively), an oscillating pattern of effluent alkalinity values was observed. This may be an expected result from a bioreactor with different biomass generation from that in Equation 2.1. The biomass generated may be used as a carbon source in the heterotrophic denitrification process and, may affect the alkalinity generation. Although the reduction of ClO_4^- produces alkalinity, it had a negligible effect on the total effluent alkalinity.

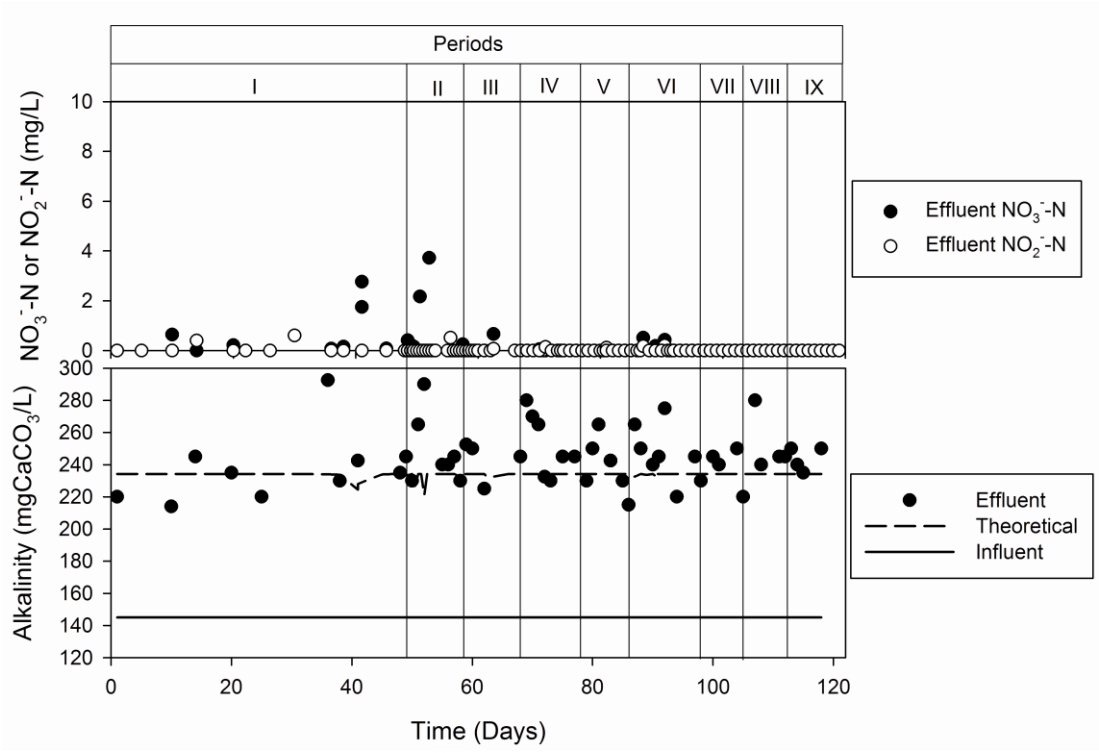

Figure 2.3 : Feed and effluent NO_3^--N, NO_2^--N and alkalinity variations of R2. Average influent nitrate concentration was 25.20±0.80 mg/L. The theoretical alkalinity concentration was calculated according to Equation 2.1.

For both reactors, batch adsorption studies revealed that ClO_4^- and NO_3^- were not adsorbed on elemental sulfur/limestone and sand particles in autotrophic and heterotrophic reactor respectively (data not shown).Effluent average dissolved organic carbon was 2.40 mg/L in the first three periods. After the third period, influent was supplemented with perchlorate, which did not significantly affect the carbon removal performance of the bioreactor throughout the operation. The effluent average DOC concentration in period 4 was 2.40 mg/L. When the highest perchlorate concentration (1000 μg/L) was added to the feed in the last period, the effluent DOC concentration averaged 4 mg/L. On some occasions, effluent DOC concentrations increased to 7.5 mg/L, which may be due to sloughing of biofilm and the release of extracellular polymeric substances and soluble microbial products to the liquid phase.

2.4 Discussion

The heterotrophic reactor performed better than the autotrophic reactor for complete nitrate and perchlorate reduction. In Southern California, approximately 395 wells in 96 water bodies were reported to contain perchlorate in the range 3.3–820 μg/L

(Tikkanen, 2006). Similarly, other places in the USA, as well as in India, the perchlorate concentration in water resources ranged between 0.02 and 1000 μg/L (Ye et al., 2012). For periods 4–9, the average effluent perchlorate concentration in the autotrophic denitrification column was 56±47.08 μg/L. Sulfur was the sole electron source, with a low solubility of 5 μg/L at 20 °C (Ju et al., 2007). Therefore, nitrate and perchlorate compete for the same electron source, and most of the electrons are used for nitrate removal. Equations 2.1 and 2.2 can be used to calculate the fractions of electrons used for denitrification and for perchlorate reduction. Even when the highest perchlorate concentration of 1000 μg/L is considered, around 99.6% of the electrons generated from elemental sulfur were used for denitrification, while the remaining 0.4% was used for perchlorate reduction. Ziv-El and Rittmann studied simultaneous nitrate and perchlorate reduction using H_2 as an electron acceptor in an H_2-based membrane biofilm reactor (Ziv-El & Rittmann, 2009). They reported that when the electron donor availability is limited, electron acceptors had a clear hydrogen utilization order of oxygen, nitrate, nitrite and perchlorate. Similar results were also reported by other authors for organic donors. Choi and Silverstein reported that when 10 mg NO_3^--N/L was added to a perchlorate reducing plug flow reactor, effluent perchlorate concentrations were 14±31 μg/L at an influent perchlorate concentration of 1000 μg/L. Further increasing NO_3^--N to 16 mg/L resulted in an average effluent perchlorate concentration of 19 μg/L (Choi & Silverstein, 2008).

The perchlorate reduction performance of autotrophic and heterotrophic processes was compared under steadily increasing loading rates, by decreasing the HRT or increasing influent perchlorate concentration. The perchlorate reduction rates for autotrophic and heterotrophic reactors were 12 and 24 mg/(L.d), respectively. Ju et al. reported perchlorate reduction rates as >36.60, 17.80 and 8.41 mg/L.d when H_2, S^0 and Fe^0, respectively, were used as electron sources(Ju et al., 2008).

Although, theoretically, 2.47 mg CH_3OH is required to denitrify each mg of nitrate nitrogen (Equation 2.1), generally higher levels of methanol were required, which may be due to higher biomass generated than expected. The methanol requirement for heterotrophic denitrification was reported to be 2.65±0.3 mg methanol/mg NO_3^--N in the study by Sahinkaya and Kilic (Sahinkaya & Kilic, 2014). Similarly, this ratio was reported as 2.72 mg methanol/mg NO_3^--N in another study (Sahinkaya, Dursun, et al., 2011). In the present study, the reactor was operated under nitrate

limiting conditions with a methanol/NO_3^--N ratio of 4:1 to evaluate the potential of heterotrophic processes to guarantee highly efficient nitrate reduction and simultaneously reduce perchlorate. Although operating the reactor under nitrate limiting conditions may guarantee high nitrate reduction efficiency, unused organic substrate may contaminate the effluent, potentially requiring further treatment. In our study methanol was observed in the effluent up to around 20 mg/L. Therefore, although methanol-based denitrification is fast and effective, a sulfur-based autotrophic denitrification process may be preferred due to elimination of effluent contamination by organic substrate. Although the heterotrophic reactor had a better perchlorate reduction, methanol utilization causes several risks such as bacterial growth in water distribution networks and production of disinfection byproducts. Although an elemental sulfur-based denitrification process could not achieve complete perchlorate reduction, the use of granular S^0 offers great advantages. Besides decreasing organic residual risk, continuous dosing is not required. While it could be used as reactor supporting media, it also provides a limitless electron source in the reactor.

The observed maximum nitrate removal rate in the autotrophic reactor was 0.3 g NO_3^--N/(L.d). The efficiency of the autotrophic process was comparable with that of heterotrophic denitrifying processes utilizing methanol as carbon source and receiving 20 mg NO_3^--N/L at a HRT of 5.3 h, which had a nitrate removal efficiency of 88.8% (Wasik et al., 2001). Sierra-Alvarez et al. obtained a maximum denitrification rate of 0.25–0.3 g NO_3^--N/(L.d) in a lab-scale packed-bed bioreactor with a sulfur/limestone ratio of 1:1 (Sierra-Alvarez et al., 2007). Similarly, Soares obtained a denitrification rate of 0.2 g NO_3^--N/(L.d) using a packed bed-bioreactor filled with sulfur granules only(Soares, 2002). Sahinkaya et al. obtained a maximum denitrification rate of 0.2 g NO_3^--N/(L.d) in a lab-scale sulfur-packed bioreactor receiving simulated groundwater(Sahinkaya, Dursun, et al., 2011). In the study by Kimura et al., complete denitrification of 25 mg NO_3^--N/L was attained in an autotrophic membrane bioreactor (MBR) at a HRT of 160 min corresponding to a denitrification rate of 0.22 g NO_3^--N/(L.d) (Kimura, Nakamura, & Watanabe, 2002). In the present study, a maximum denitrification rate of approximately 0.3 g NO_3^--N/(L.d) was obtained, which is at least as high as the denitrification rates obtained in other studies.

Effluent sulfate concentration was affected by the influent sulfate concentration and denitrified nitrate nitrogen. Influent average sulfate concentration was 35.40±1.40 mg/L and the expected sulfate production in the case of complete denitrification would be 184±7.40 mg/L. Theoretical sulfate production, calculated by the sum of influent and produced sulfate concentrations, was 220±7.20 mg/L, which is quite close to the average measured sulfate concentration, 224±50.20 mg/L.

The presence of other oxidized anions may inhibit biological perchlorate reduction. The inhibition of perchlorate reduction in the presence of nitrate was attributed mainly to the suppression of the perchlorate reductase enzyme by nitrate. In a study conducted by Ghosh et al., perchlorate and nitrate were reduced simultaneously with organic electron donors (Ghosh et al., 2011). A single perchlorate reductase may catalyze the reduction of both nitrate and perchlorate (Bardiya & Bae, 2011). On the other hand, the existence of separate enzymes for nitrate and perchlorate reduction had been proposed for *A. Auillum* strain PS 33 and strain Perc1ace (Okeke et al., 2002). However, it was found that bacteria grown in the presence of both nitrate and perchlorate had a better perchlorate reduction than bacteria grown with perchlorate only (Xu, Trimble, Steinberg, & Logan, 2004). Although perchlorate reduction was inhibited in the presence of nitrate, its reduction was accelerated immediately after the complete nitrate reduction. This competitive inhibitory effect has been reported by many researchers in the literature during autotrophic (London et al., 2011) and heterotrophic processes (Wang et al., 2012). London et al. reported that a 90 h retention time is required when nitrate is present, whereas only 10 h was required to reduce perchlorate in the absence of nitrate (London et al., 2011). Despite inhibitory effects, nitrate may be required for perchlorate reduction, as low perchlorate concentration may not sustain bacterial growth and nitrate may be required as primary electron acceptor. Nerenberg et al. experimentally determined the minimum perchlorate concentration to support bacterial growth to be 14 μg/L(Robert Nerenberg et al., 2006).

Nitrite as an intermediate product of the denitrification process was not detected for either reactors. This is most probably due to an abundance of electron donor availability. Nitrite accumulation is reported when electron donors are limited (Ziv-El & Rittmann, 2009). Intermediate products (ClO_2^- and ClO_3^-) of perchlorate reduction were also not observed, as none of these intermediates have been reported

to accumulate in the system (Ghosh et al., 2011). Although heterotrophic reduction is fast and stable, the effluent may be contaminated with organic substrate. To solve this problem, Sahinkaya and Dursun developed a mixotrophic process, combining the heterotrophic and S^0 based autotrophic processes. By this method, (1) excess sulfate production was prevented, (2) the alkalinity requirement of the autotrophic process may be supplied by a heterotrophic process, and (3) the risk of effluent contamination with organic substrates may be eliminated (Sahinkaya & Dursun, 2012). Alternatively, Huang et al. developed an acetic acid–FeS based heterotrophic–autotrophic sequential process. The process has several advantages: (1) effluent from the heterotrophic reactor pumped to a FeS-based autotrophic reactor yields better effluent quality because residual nitrate, nitrite and remaining acetic acid are removed; (2) the FeS-based autotrophic denitrification has lower acidity and sulfate production: and (3) other toxic metals could also be absorbed by FeS (Huang, Chi, Chen, & Shi, 2011). Although requiring further research, it is possible that a similar mixotrophic denitrification process may also be used to decrease perchlorate concentration below 5 μg/L without contaminating treated effluent with unused organic substrate.

2.5 Conclusions

Although nitrate competitively inhibited perchlorate reduction, both anions were simultaneously removed using sulfur-based autotrophic and heterotrophic processes. Even at nitrogen loading rates of 300 and 600 mg NO_3^--N/(L.d), autotrophic and heterotrophic denitrifying bioreactors experienced complete denitrification. The perchlorate in the heterotrophic reactor was also completely reduced at influent concentrations of 50–1000 μg/L. The autotrophic reactor could also reduce perchlorate by 95% at an influent perchlorate concentration of 1000 μg/L. In the autotrophic reactor, nitrate was the preferred electron acceptor, and perchlorate reduction may be adversely affected by nitrate presence. Hence, the autotrophic reduction performance may be an electron source limited due to the slow and low dissolution of elemental sulfur. The effluent perchlorate concentration in autotrophic denitrification process varied between 20.88 and 84.96, independent of influent perchlorate concentrations of 50–1000 μg/L.

3. EVALUATION OF NITRATE AND PERCHLORATE REDUCTION USING SULFUR-BASED AUTOTROPHIC AND MIXOTROPHIC DENITRIFYING PROCESSES

3.1 Introduction

Elemental sulfur or elemental sulfur/methanol-based up flow denitrifying bioreactors have been shown to simultaneously reduce multiple oxidized contaminants (Akunna, Bizeau, & Moletta, 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. (Jinwook Chung, Nerenberg, & Rittmann, 2007; Jinwook Chung, Rittmann, et al., 2007).

Perchlorate (ClO_4^-) 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) perchlorate is usually present in water sources in μg/L level hampering 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_4^- (Fox, Oren, Ronen, & Gilron, 2014; Ju et al., 2008). However, all bioreactor concepts studied to date rely on the continuous addition of an electron-donating substrate.

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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 proper amount of organic carbon. Heterotrophic denitrification with methanol as the electron donor is shown in Equation (3.1). The equation shows that 2.47 g methanol is required for complete reduction of 1 g NO_3^--N to N_2 gas (Sahinkaya & Dursun, 2012).

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (3.1)$$

Acetate is the most commonly used electron donor in the 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 3.2 and 3.3 show basic perchlorate reduction with hydrogen (R Nerenberg & Rittmann, 2004) and acetate (Nor et al., 2011), respectively, as electron sources.

$$ClO_4^- + 4H_2 \rightarrow Cl^- + 4H_2O \quad (3.2)$$

$$CH_3COOH + ClO_4^- \rightarrow 2CO_2 + Cl^- + 2H_2O \quad (3.3)$$

The ratio of 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) and 93% perchlorate (from 100 to 7± 0.8 μg/L) reduction with mixed anoxic culture in the presence of ethanol (Ricardo, Carvalho, Velizarov, Crespo, & Reis, 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). Without enough added carbon, nitrite, which is more toxic than nitrate, may accumulate. To overcome these problems, autotrophic perchlorate reducing bioreactors with inorganic electron donors such as H_2 (Ziv-El & Rittmann, 2009),

Fe^0 (Cao, Elliott, & Zhang, 2005) and elemental sulfur (Ju et al., 2008) have also been used. Cao et al., (2005) investigated 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 was favorable in terms of thermodynamics (activation energy was calculated as 79.02 ± 7.75 kJ/mole), perchlorate reduction was limited by the slow kinetics (Cao et al., 2005). Ju et al., (2008) tested the performance of various inoculums taken from aerobic or anaerobic environments with various electron donors. The reduction rate was 0.18 mM/d with S^0 and aerobic process sludge as the electron donor and inoculum respectively. Reduction rates of hydrogen and Fe^0 with the same inoculum were ≥0.37 mM/d on day 8 and 0.085 mM/d 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^0 provides a slow release of electrons on demand, eliminating dose adjustment in a simple and reliable operation. The expected stoichiometry of the reaction is as follows (Sahinkaya & Dursun, 2012):

$$55S^0 + 50NO_3^- + 38H_2O + 20CO_2 + 4NH_4^+ \rightarrow 4C_5H_7O_2N + 55SO_4^{2-} + 25N_2 + 64H^+ \quad (3.4)$$

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 the C/N ratio was 2:1, 30 mg NO_3^--N/L was completely reduced without excess sulfate production (<130 mg/L) (Liu et al., 2009). Autotrophic, mixotrophic and heterotrophic denitrification performances were compared by Oh et al. (2001). While the denitrification rate for their sulfur-based reactor was 1.4 kg/(m^3.d), it increased to 1.92 and 2.7 kg/(m^3.d) with 132.8 and 571.4 mg/L methanol supplementation (Oh et al., 2001). Sahinkaya and Dursun, (2012) reported that the acidity produced by the S^0 based autotrophic reactor was neutralized by the alkalinity produced by heterotrophic process and that complete reduction of 75 mg NO_3^--N/L 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 S^0/methanol-based mixotrophic process. A complete reduction of 75

mg NO_3^--N/L and 10 mg/L Cr(VI) were achieved under varying C/N ratios (1.33–2) and with a HRT of 3.7 (Sahinkaya et al., 2013).

Perchlorate concentrations in drinking water sources are relatively low (micrograms per liter) 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 also provides the satisfying sulfate and alkalinity concentrations in the effluent. This study aims to compare the simultaneous nitrate and perchlorate bioreduction performances of elemental sulfur-based autotrophic and mixotrophic denitrifying 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.

3.2 Materials and Methods

3.2.1 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, and 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). Small sulfur and limestone particles were used so as to not limit the denitrification rate because 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 an up-flow mode using adjustable peristaltic pumps at 28–30 °C in a temperature controlled room.

3.2.2 Inoculation and Operation of the Reactors

A 30 ml (VSS=26000±430 mg/L) 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 autotrophic reactor. Around 30 ml (VSS=16000±240 mg/L) denitrifying sludge obtained from another mixotrophic reactor was used for the inoculation of mixotrophic bioreactor. The reactors were operated in batch mode for first 3 days after inoculation and then were

operated continuously up-flow mode. The freshly prepared feed solution was deoxygenated by passing through N_2 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 K_2HPO_4 as a source of phosphorus, and KNO_3 to obtain 25 mg NO_3^--N/L. To supply alkalinity in the autotrophic reactor, 375 mg $NaHCO_3$/L was added to the feed. The mixotrophic reactor was supplemented with methanol (25 and 35 mg/L) to provide an external carbon source. Operational conditions of the reactors were presented in Table 3.1.

Table 3.1 : Operational conditions of autotrophic and mixotrophic reactor (influent contained 25 mg/L NO_3^--N and 50 mg/L K_2HPO_4 for all periods).

Mixotrophic Reactor	Periods								
	1	2	3	4	5	6	7	8	9
Days	0-34	35-47	48-58	59-70	74-102	103-115	116-129	130-143	145-174
NO_3^--N loading rate (mg N $l^{-1}d^{-1}$	50	150	400	400	400	400	400	400	400
CH_3OH (mg/L)	-	-	25	25	25	25	25	35	35
ClO_4^- (µg l^{-1})	-	-	-	50	100	200	300	500	1000
Alkalinity (mg $CaCO_3$/L)	130	120	105	108	130	140	125	120	120
HRT (h)	12	4	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Autotrophic Reactor									
Days	0-295	296-305	306-312	313-326	327-333	333-344	345-356	357-363	364-369
NO_3^--N loading rate (mg N $l^{-1}d^{-1}$	50	150	300	300	300	300	300	300	300
ClO_4^- (µg l^{-1})	-	-	-	50	100	200	300	500	1000
Alkalinity (mg $CaCO_3$/L)	390	495	495	495	495	495	495	495	495
HRT (hours)	12	4	2	2	2	2	2	2	2

Both reactors were operated under varying operational (Table 3.1) 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 NO_3^--N, NO_2^--N, ClO_4^-, ClO_3^-, ClO_2^-, Cl^-, SO_4^{2-}, HS^-, dissolved organic carbon (DOC), pH and alkalinity. The feed solution was also sampled regularly for the determination of NO_3^--N, ClO_4^-, Cl^-, NO_2^--N, NO_2^--N, DOC, pH, and alkalinity.

3.2.3 Batch Adsorption Experiments

Batch adsorption experiments were conducted to identify NO_3^- and ClO_4^- removal mechanisms. In the experiments, 100 ml serum bottles were supplemented with 25 mg NO_3^--N/L and 1,000 μg ClO_4^-/L for the autotrophic denitrification experiments, serum bottles were supplemented with 1.5 g elemental sulfur. Similarly, for the mixotrophic denitrification experiments, serum bottles were supplemented with 1.5 g elemental sulfur, 1 g limestone and 100 mg/L CH_3OH. All serum bottles were operated in a temperature controlled room at 28–30 °C and regularly sampled for the measurements of NO_3^--N, and perchlorate.

3.2.4 Analytical methods

Chlorate, chlorite, chloride, nitrate, nitrite, and sulfate were analyzed by suppressed conductivity ion chromatography using Shimadzu HIC-SP system fitted with a DIONEX Ion-Pac AS9-HC column (4 mm× 250 mm with the detection limit of 0.1 mg/L). Perchlorate was measured by DIONEX 500 system (suppressed conductivity) with Ion- Pac AS20 column (2 μg/L detection limit). Dissolved organic carbon was measured by 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 ions and sulfide measurements.

3.3 Results and Discussions

3.3.1 Perchlorate Reduction

Both autotrophic and mixotrophic reactors simultaneously reduced nitrate and perchlorate. Perchlorate concentration in groundwater has been reported to be up to 1,000 μg/L (Ye et al., 2012), 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 at a constant nitrate loading rate of around 300 mg NO_3^--N/(L.d) (Table 3.1). In the fourth period, perchlorate concentrations in the influent and effluent of the autotrophic reactor averaged 50

µg/L and 15.61± 11.80 µg/L, respectively, corresponding to 69% removal (Figure 3.1). In the following period, removal efficiency increased to 100% (i.e. effluent perchlorate was below detection limit of 2 µg/L), except on day 328 when the effluent perchlorate concentration was 21.58 µg/L (Figure 3.1). Increasing perchlorate concentrations resulted in better perchlorate reduction efficiency and an increase in the reduction rate due to the bacterial population's acclimation to higher perchlorate loads, a finding supported by other studies (Webster, Guarini, & Wong, 2009).

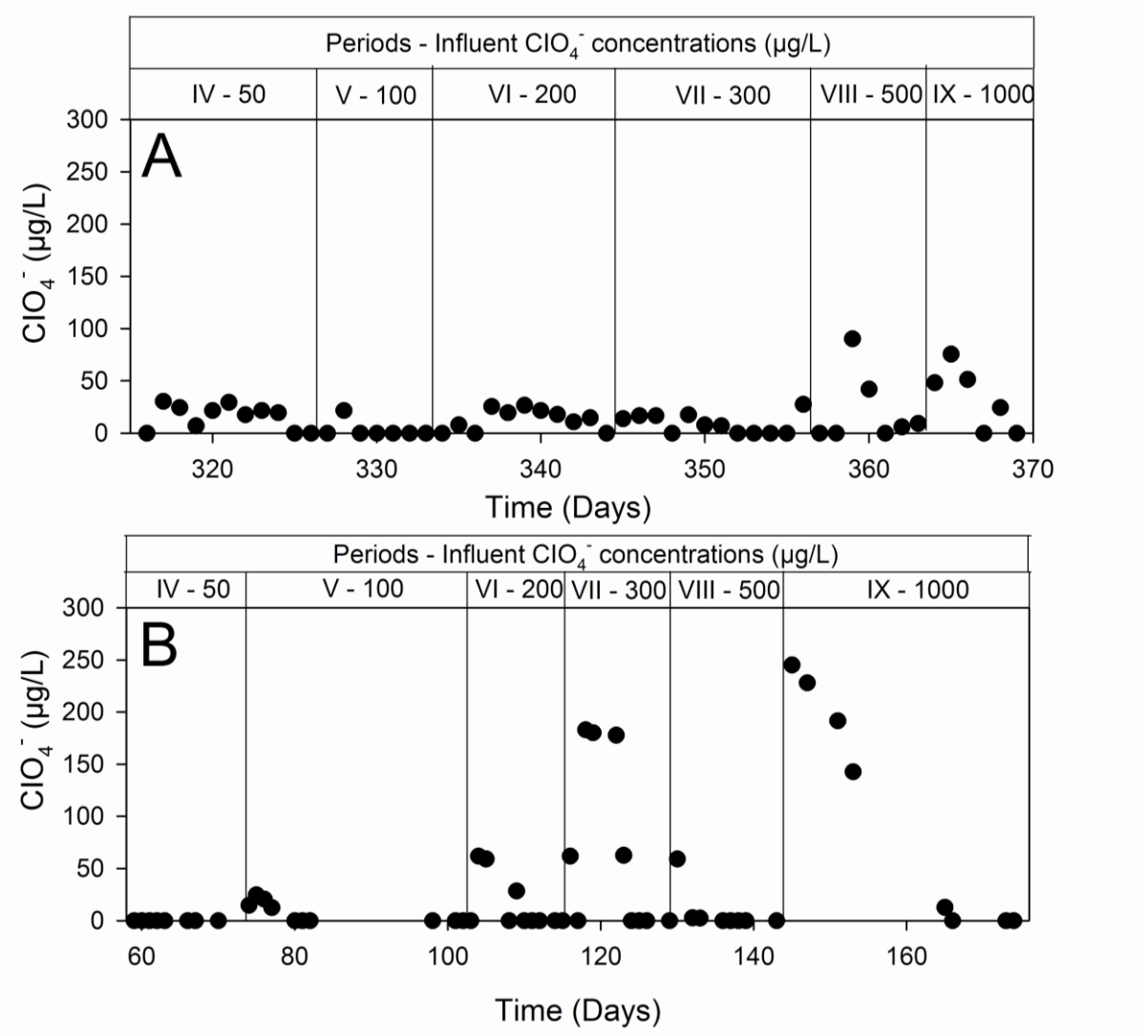

Figure 3.1 : Influent and effluent perchlorate concentrations for autotrophic (A) and mixotrophic (B) reactors.

Accordingly, when the influent perchlorate concentration in the autotrophic process was further increased to 200 µg/L in period 6 (Table 3.1), effluent perchlorate concentrations in the autotrophic bioreactor averaged 12.03±10.3 µg/L, a 94% removal efficiency. This efficiency was similar to that obtained during period 5.

Additionally, similar effluent perchlorate removal efficiencies of 97% and 96% were detected in the seventh and eight periods 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 and 33.23 ± 30.4 μg/L, respectively (Figure 3.1).

Better reduction efficiencies were observed in methanol/elemental sulfur-based mixotrophic reactor. On day 59, perchlorate was added to the influent with 50 μg/L and effluent perchlorate concentrations were always below the detection limit (2 μg/L) throughout this period. In the following period (days 74–102), the influent perchlorate concentration was increased to 100 μg/L decreasing the removal efficiency from 100 to 75%. However, perchlorate concentrations in the effluent decreased below detectable levels on day 80 and remained static until the end of this period. Further increasing the perchlorate concentration to 200 μg/L led to a performance decrease down to 69%. Similarly, in the autotrophic reactor, as the bacterial population acclimated to higher perchlorate concentrations, removal efficiencies recovered, on day 110. Although increasing perchlorate concentrations led to a decrease in perchlorate removal efficiency, the reactor recovered it's compete reduction performance (Figure 3.1). In the last period, the complete reduction of 1000 μg/L perchlorate was accomplished. Intermediate products (ClO_2^- and ClO_3^-) of perchlorate reduction were not detected throughout the autotrophic and mixotrophic reactor operation. The presence of other electron acceptors may competitively inhibit system performance. For example perchlorate reducing bacteria prefer to utilize O_2 over perchlorate and a 2 mg/L oxygen concentration could completely inhibit the perchlorate reduction in *A suillum* (Chaudhuri, O'Connor, Gustavson, Achenbach, & Coates, 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 NO_3^-/N_2 with 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, due to increased substrate availability. Increasing influent perchlorate concentration from 100 μg/L to 1000 μg/L increased the removal rate from 1,163 μg/(L.d) to 11600 μg/(L.d), respectively, in the autotrophic reactor. A similar S^0 oxidizing reactor was reported to be treating approximately 8000 mg/L perchlorate with the reduction rate of 13846 μg/(L.d) (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 1000 μg/L perchlorate together with 25 mg/L NO_3^--N was accomplished in our study with the highest perchlorate reduction rate of 16000 μg/(L.d). Higher perchlorate reductions, up to 250 mg/L, were reported for heterotrophic processes (Fox et al., 2014). In our study, the maximum perchlorate concentration fed to the bioreactor was 1000 μg/L to simulate contaminated drinking waters. Chung et al. (2007a) evaluated perchlorate reduction in a hydrogen-based membrane biofilm reactor and reported that the maximum perchlorate reduction rate was 700 μg/(L.d). Wang et al., (2013) 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 perchlorate. Nerenberg et al., (2006) found that the specific reduction rates for perchlorate and chlorate as 3100 μg/(mg DW.d) and 6300 μg/(mg DW.d) respectively. Drinking water standards of the WHO, EU or US-EPA do not include any reference concentrations for perchlorate (Blake et al., 2009). Only the EPA set a reference dose of 0.7 μg perchlorate/kg body weight corresponding to the drinking water equivalent level of 24.5 μg/L assuming that water is the only source of perchlorate consumption. The autotrophic reactor provided necessary treatment to meet with this reference dose except at last period with 1000 μg/L influent. The mixotrophic reactor gave better performance compared to the autotrophic reactor.

3.3.2 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.d) and nitrate was reduced completely except days 182 – 193 when operational problems occurred. For the rest of this period, effluent nitrate concentrations were below detection limits (<0.1 mg/L). In periods 2 and 3 nitrate loading was increased to 150 mg NO_3^--

N/(L.d) and 300 mg NO_3^--N/(L.d), respectively. In these periods effluent nitrate concentrations were below 2 mg NO_3^--N/L. For periods 2 and 3, average effluent nitrate concentrations were 0.33 ± 0.7 and 0.54 ± 1.1 mg NO_3^--N/L. Effluent nitrite concentrations were below detection limits during these periods except on day 300 when there was a concentration of 0.04 mg NO_2^--N/L (Figure 3.2).

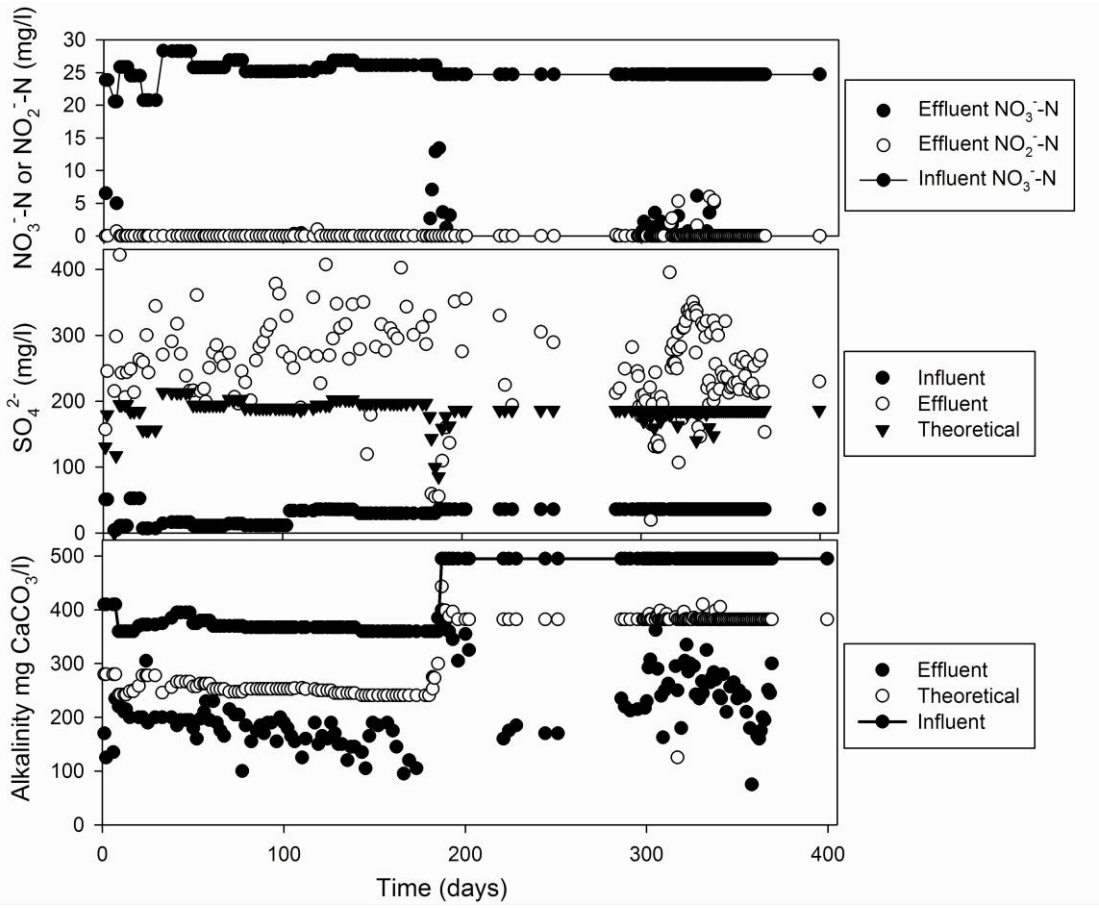

Figure 3.2 : Feed and effluent NO_3^--N, NO_2^--N, sulfate and alkalinity variations of the autotrophic reactor. Theoretical sulfate concentration was calculated according to Equation 3.4.

Perchlorate (50 µg/L) was added to the feed in period 4 (day 316) and its concentration was increased gradually to 1000 µg/L (Table 3.1) at a constant nitrate loading rate of around 300 mg NO_3^--N/(L.d). The addition of perchlorate did not affect the denitrification performance adversely (Figure 3.2). In periods 4 and 5, when influent perchlorate was 50 µg/L and 100 µg/L, effluent nitrate concentrations averaged 0.41 ± 1.48 mg NO_3^--N/L and 0.07± 0.48 mg NO_3^--N/L, respectively. Influent perchlorate concentrations were further increased to 200, 300, 500 and 1000 µg/L in 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 (ignoring days 182–193 during which operational problems occurred). In periods 3–9, nitrate reduction rates were around 300 NO_3^--N/(L.d), 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.d) (Sahinkaya et al., 2013). Sahinkaya and Dursun, (2012) also obtained the denitrification rates of 450 mg/(L.d) and 300 mg/(L.d) for mixotrophic and autotrophic processes respectively.

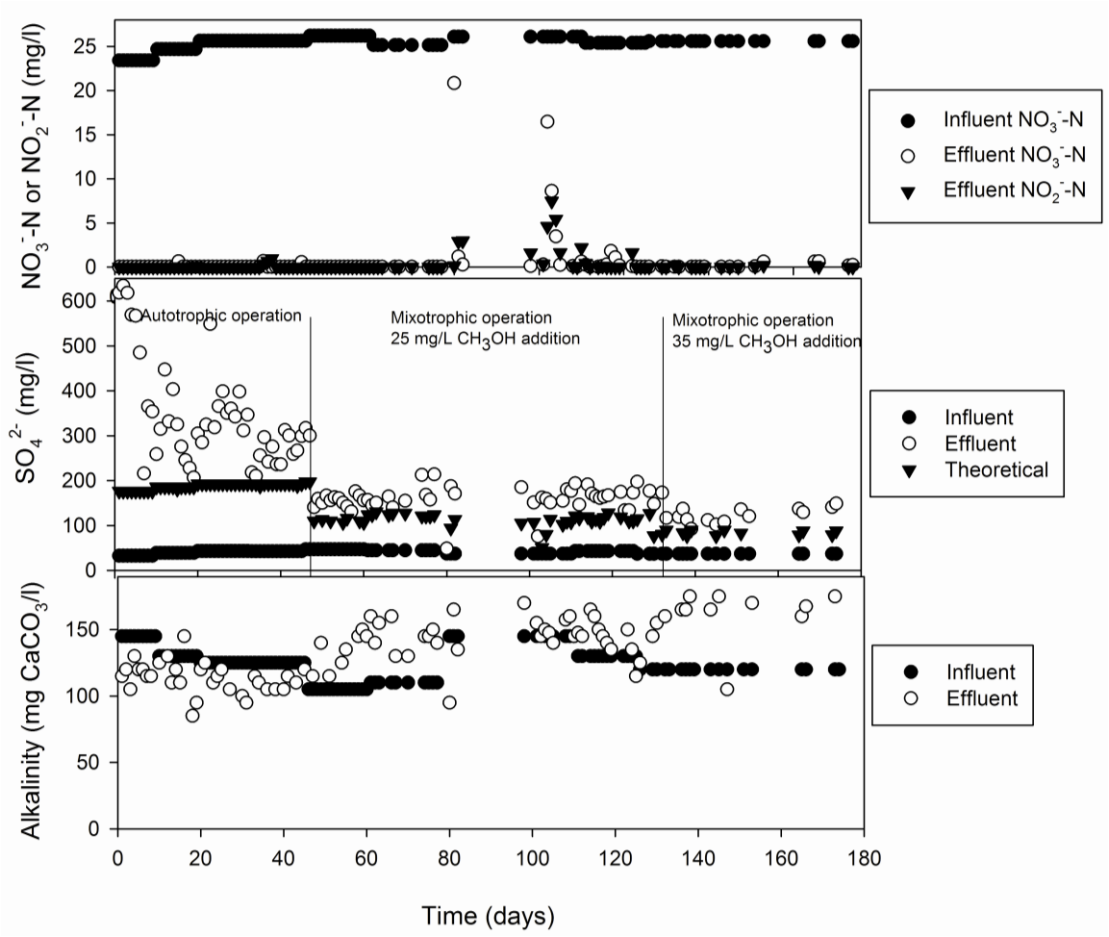

Figure 3.3 : Feed and effluent NO_3^--N, NO_2^--N, sulfate and alkalinity variations of mixotrophic reactor. Theoretical sulfate concentration was calculated according to Equation 3.4.

The mixotrophic reactor was operated for 174 days under 9 different operational periods (Table 3.1). In the first period, the nitrate load was 50 mg/(L.d) and it increased to 150 mg/(L.d) and then 400 mg/(L.d) in periods 2 and 3, respectively (Figure 3.3). Complete nitrate reduction (25 mg NO_3^--N/L) was achieved in the first period. When the nitrate load was increased to 150 mg/(L.d), the effluent nitrate concentration was almost under the detectable level, however nitrite (<1 mg/L) was detected for the first three days of the second period. 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. The fraction of nitrate denitrified by heterotrophs was determined indirectly from the production of sulfate (Oh et al., 2001). According to Equation (3.1), 2.47 mg methanol is required to reduce each mg NO_3^--N, so the addition of 25 mg/L methanol stimulates the heterotrophic reduction of around 10 mg NO_3^--N. Thus, a decrease in sulfate production of 75.4 mg was 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, corresponding to around 8 mg/L NO_3^--N heterotrophically reduced. Similarly, when the methanol in the feed was further increased to 35 mg/L in period 8, effluent sulfate concentration decreased to 142.10± 20.90 mg/L corresponding to an increase in the concentration of heterotrophically reduced NO_3^--N from 8 to 12.5 mg/L. Throughout the study, average effluent nitrate concentration was 0.20 ± 0.9 mg NO_3^--N/L. Complete reduction of 25 mg NO_3^--N/L was achieved and the maximum nitrate reduction rate for autotrophic and mixotrophic reactors was 300 mg NO_3^--N/(L.d) and 400 mg NO_3^--N/(L.d), respectively. Batch adsorption studies revealed that ClO_4^- and NO_3^- were not adsorbed on elemental sulfur/limestone in either kind of reactors (data not shown). The mixotrophic reactor was operated under methanol limiting conditions, the 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 mg heterotrophically removed nitrate was 2.73±1.75 during periods 3–9. According to Equation 3.1, theoretically 2.47 mg CH_3OH is required to denitrify each mg NO_3^--N. However, a generally higher methanol requirement has been reported, perhaps due to a higher biomass generation than predicted by Equation 3.1. The methanol requirement for heterotrophic denitrification was reported as 2.65±0.3 mg methanol/mg NO_3^--N in a study by Sahinkaya and Kilic, (2014). Similarly 2.72 (Sahinkaya, Dursun, et al., 2011) and 3.06 (Sahinkaya et al., 2013) were the other reported methanol/NO_3^--N ratios in denitrifying reactors.

3.3.3 Sulfate Production

According to Equation (3.2), 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 were presented in Figure 3.2. An average

theoretical effluent sulfate concentration of 215±23.60 mg/L, whereas effluent sulfate concentrations from the autotrophic reactor averaged 259±87.70 mg/L throughout the study. The average sulfate concentration of tap water was 30±10.60 mg/L. Although precautions were taken (e.g. nitrogen gas bubbling of feed after preparation and keeping the feed in a collapsible container), high effluent sulfate concentrations could be caused by the leakage of oxygen during sampling or feeding. The mixotrophic reactor was supplemented with methanol in the period 4 at a concentration of 25 mg CH_3OH/L. Addition of methanol decreased average sulfate production from 238.20±36.40 to 176.10±11.50 mg/L. Sulfate production decreased significantly after methanol addition. The fast process response was probably because sludge used for inoculation was taken from another mixotrophic reactor reducing nitrate. In the period 8, increasing the methanol in the feed to 35 mg/L led to a further decrease in sulfate concentration to 150.25±16 mg/L. Considering the sulfate generation in the case of 25 mg and 35 mg methanol supplementations, it was calculated that around 67% and 53% NO_3^--N was autotrophically denitrified, respectively. Average effluent sulfate concentration was below the US-EPA, EU, and Turkish drinking water standard of 250 mg/L. According to these results, the sulfate concentration in the effluent of the reactor can be controlled by external carbon supplementation.

3.3.4 Alkalinity

The effluent pH and alkalinity concentrations of autotrophic reactor were lower than those of influent due to acid generation in autotrophic processes (Equation (3.4)). While average influent alkalinity was 443±62 mg $CaCO_3$/L, it decreased to 215±63 mg $CaCO_3$/L in the effluent. Sulfur-based autotrophic denitrification of each mg NO_3^--N would consume 4.57 mg $CaCO_3$. Therefore, denitrification of 25 mg NO_3^--N would consume 114 mg $CaCO_3$. The average effluent alkalinity concentration can be calculated as around 329±66 mg $CaCO_3$/L, much higher than the measured concentrations. This result indicates that acidity may also be produced due to the oxidation of elemental sulfur with the oxygen leaking into the reactor with the 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 during period 3 and 4 as well. In the period 4 and during 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 $CaCO_3$/L in periods 1 and 2, at an influent alkalinity concentration of 125.60±11.50 mg/L. In the third period, the addition of methanol promoted the heterotrophic denitrification and alkalinity production. For periods 3–7, almost complete denitrification was achieved at 25 mg NO_3^--N/L. Average NO_3^--N concentration reduced by the heterotrophic process was 8.25±0.24 mg/L in periods 3–7. In the heterotrophic denitrification, theoretically 3.57 mg $CaCO_3$/L is produced for each mg NO_3^--N denitrified (Equation (3.1)) (Oh et al., 2001). Similarly, 4.57 mg $CaCO_3$ is utilized in autotrophic denitrification for each mg NO_3^--N. Considering that 8.25 mg NO_3^--N is denitrified by heterotrophs, the whole system needed 47 mg $CaCO_3$. Limestone dissolution was another factor providing alkalinity to the system. Influent and effluent alkalinity concentrations during periods 3–7 (when influent methanol concentration was 25 mg/L) were 122.50±16 mg $CaCO_3$/L and 143.60±14.60 mg $CaCO_3$/L, respectively. When methanol was further increased to 35 mg/L, influent and effluent alkalinity concentrations were 120 ±12 and 161± 28 mg $CaCO_3$/L, respectively.

3.3.5 Residual Organics

Methanol as an organic carbon source was added to the mixotrophic reactor during periods 3–9. Dissolved organic carbon was almost completely removed in the bioreactor. Influent methanol concentrations were 25 mg/L and 35 mg/L 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 (Figure 3.4).

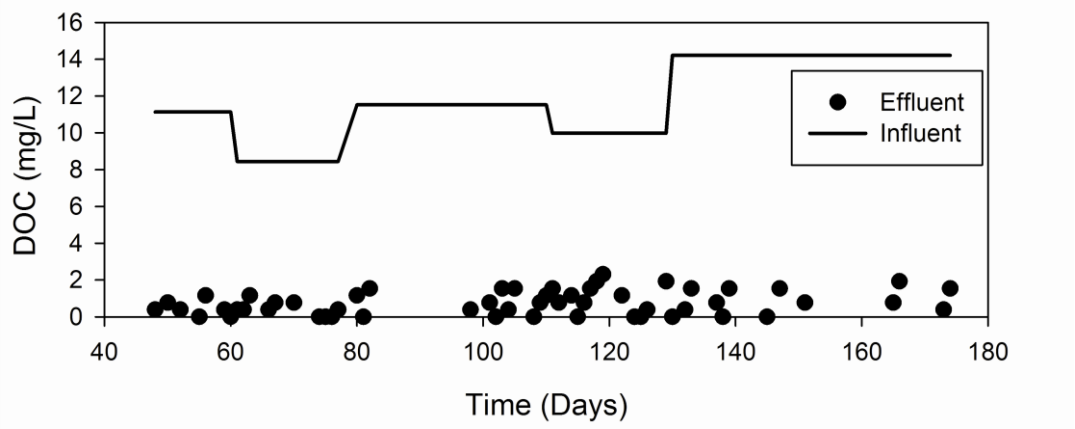

Figure 3.4 : Feed and effluent dissolved organic carbon variations in the mixotrophic reactor.

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 during disinfection processes. Additionally, a poor C/N ratio leads to improper denitrification and leads to accumulation of nitrite or extra production of nitrous other than nitrogen gas (Kim, Nakano, Lee, Kanchanatawee, & Matsumura, 2002). A combination of an elemental sulfur based autotrophic process with a methanol-based heterotrophic process could overcome all of these drawbacks. Proper dosing of methanol to autotrophic denitrifying reactor could completely remove nitrate and perchlorate without excess sulfate or DOC contamination in the effluent.

3.4 Conclusions

Complete removal of nitrate was achieved in autotrophic and mixotrophic reactors with loading rates of 300 mg NO_3^--N/(L.d) and 400 mg NO_3^--N/(L.d), respectively. Perchlorate was decreased from 1000 to 33.23±30.4 μg/L 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. Effluent sulfate concentrations of 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 supplementing methanol provided many advantages, including (1) increasing the perchlorate removal rate (2) lowering the effluent sulfate concentration, and (3) completing the nitrate reduction under nitrate loading rates of 400 mg N/(L.d).

4. HETEROTROPHIC-AUTOTROPHIC SEQUENTIAL SYSTEM FOR REDUCTIVE NITRATE AND PERCHLORATE REMOVAL

4.1 Introduction

Perchlorate is a soluble anion that consists of a chlorine atom surrounded by four oxygen atoms in a tetrahedral array. It has been used in rocket propellants, highway safety flares, air bag inflators, fireworks and matches (Motzer, 2001; Raj & Muruganandam, 2013). Because of its low adsorption properties and its recalcitrant structure, it can easily reach ground water and remain for a long period due to its recalcitrant structure. In many parts of the world, extensive perchlorate contamination of the surface and groundwater was detected in 1997. At that point in time, the development of instrumental techniques that use highly sensitive chromatography, allowed the detection of levels less than 4 μg/L. These techniques demonstrated that the real extent of perchlorate contamination had been previously underestimated (Collette et al., 2003; Motzer, 2001). In many parts of the world, the presence of perchlorate has been detected in measurable quantities in rice, fish and dairy products, vegetables and even breast milk (Srinivasan & Sorial, 2009; Susarla, Bacchus, Harvey, & McCutcheon, 2000). Trace quantities of perchlorate have even been measured in rainwater (Dasgupta et al., 2005).

Perchlorate competitively inhibits the uptake of iodide by the thyroid gland resulting in the decrease in thyroid hormones. These hormones regulate the basal metabolism that is essential for normal fetal and postnatal neurological development. Currently, the United States Environmental Protection Agency (US-EPA), World Health Organization (WHO) and TSS266 (Turkish Standard Institution, standard of water intended for human consumption) do not have any limitations for perchlorate (Blake et al., 2009). Some states in the USA have already established advisory levels for

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perchlorate such as California (6 μg/L), Massachusetts (2 μg/L) and Texas (4 μg/L)(Srinivasan & Sorial, 2009).

Nitrate is another contaminant that may be present in groundwater with perchlorate. Nitrate can cause methemoglobinemia when ingested by infants and when transformed into nitrosoamines has a well-defined role in carcinoma, malformation and mutation(Della Rocca et al., 2007). Some wells in Harran Plain, Sanliurfa/Turkey contain nitrate levels as high as 180 mg NO_3^--N/L. The average concentration of whole plain was 35 mg NO_3^--N/L (Yesilnacar et al., 2008). The US-EPA sets maximum contaminant levels of 10 mg/L NO_3^--N and 1.0 mg/L NO_2^--N for drinking water (K. C. Lee & Rittmann, 2002). Several methods have been reported for the removal of nitrate and perchlorate such as adsorption, membrane filtration and electrochemical reduction. Among them biological reduction was reported as the most promising method due to its fast reaction kinetics (Srinivasan & Sorial, 2009).

Biological reduction of these anions is possible with either organic or inorganic electron donors. Heterotrophic nitrate reduction in the presence of methanol is illustrated in Equation (4.1). Similar to nitrate reduction, perchlorate can also be reduced heterotrophically in the presence of suitable organic matter. Several organic molecules can be used as carbon and electron sources for heterotrophic denitrification including methanol, acetate, lactate, pyruvate, casamino acids, fumarate, succinate, ethanol, fructose, cellobiose, mannose, xylose, pectin, nalkanes, 1-hexene and liquefied petroleum gas (Bardiya & Bae, 2011). The reduction efficiency highly depends on the chemical oxygen demand (COD)/perchlorate ratio. The optimum COD/perchlorate ratio was reported between 1.45 and 1.65 g COD/g ClO_4^- (Nor et al., 2011; Wang et al., 2013).

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (4.1)$$

Although utilization of organic electron donors is fast and effective, organic contamination risk of drinking water is of concern since the addition of exactly the right amount of organic donor is difficult to achieve (Oh et al., 2001). To overcome these problems, bioreactors utilizing inorganic electron donors such as H_2,(R Nerenberg & Rittmann, 2004) iron (Son et al., 2006) or elemental sulphur (Demirel et al., 2014; Lv, Shao, Li, & Xie, 2014; Sahinkaya et al., 2015) have been proposed.

In the sulphur-based denitrification process, elemental sulphur serves as the inorganic electron donor (Equation (4.2)). The low solubility of elemental sulphur provides a slow release of electrons on demand (Ahmed et al., 2012; Kilic et al., 2014). According to Equation (4.3), 2.51 g S^0 is required to reduce each gram of NO_3^--N. For perchlorate reduction, 0.99 g S^0 is required to reduce each gram of ClO_4^- to chloride (Equation (4.2)). Stoichiometric perchlorate and nitrate reduction in the presence of elemental sulphur are presented in Equations (4.2) and (4.3), respectively

$$2.9\ S^0 + 3.3H_2O + ClO_4^- + 1.8CO_2 + 0.46HCO_3^- + 0.46NH_4^+ \rightarrow 5.7H^+ + 2.9SO_4^{2-} + Cl^- + 0.46C_5H_7O_2N \quad (4.2)$$

$$55\ S^0 + 50NO_3^- + 38H_2O + 20CO_2 + 4NH_4^+ \rightarrow 4C_5H_7O_2N + 55SO_4^{2-} + 25N_2 + 64H^+ \quad (4.3)$$

The use of elemental sulphur offers some advantages: (1) it eliminates the risks associated with organic electron addition, (2) it's low maintenance as continuous addition of electron donors is not required and (3) it is less resource dependent because a long term supply of electron donor is stored in the insoluble S^0 particles (Ju et al., 2007). However, acidity and sulphate production are drawbacks of the autotrophic elemental sulphur-based denitrification process.

Combining the use of organic and inorganic electron donors offers many advantages such as lower acidity and sulphate production together with eliminating or reducing organic contamination risk. Elemental sulphur/methanol-based mixotrophic processes were reported successful in controlling effluent sulphate and alkalinity (Sahinkaya & Dursun, 2012; Sahinkaya, Dursun, et al., 2011). Sequential heterotrophic–autotrophic processes may offer simultaneous treatment of nitrate and perchlorate while eliminating or decreasing the contamination of treated water by unused organic matter. In the sequential process, while the heterotrophic process with a low C/N ratio reduces only a certain portion of nitrate and perchlorate, the rest could be trapped by the subsequent autotrophic reactor. Therefore, the main objective of this research is to investigate the treatment performance of the heterotrophic–autotrophic sequential process for simultaneous nitrate and perchlorate reduction. By using this sequential process, effluent organic contamination, sulphate and acidity formation can be decreased, which may increase the possibility of the process to be used in real-scale applications.

4.2 Materials and Methods

4.2.1 Denitrifying Column Bioreactors

Two denitrifying up-flow column bioreactors were operated in series; first the heterotrophic then the autotrophic reactors were used (Figure 4.1). Methanol and elemental sulphur were used as electron sources in the heterotrophic and autotrophic bioreactors, respectively. Total and working volumes of the glass reactors (R1 and R2) were 550 and 400 mL, respectively.

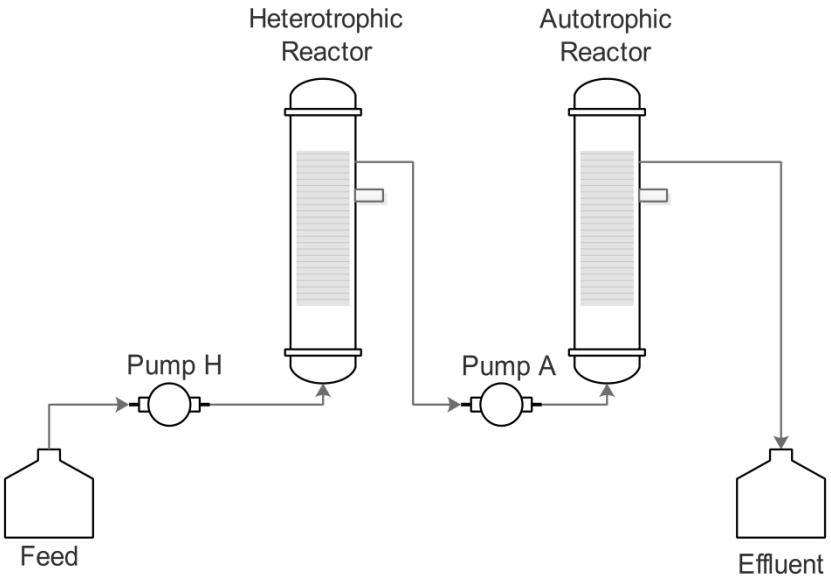

Figure 4.1 : Schematic view of heterotrophic - autotrophic sequential process.

The heterotrophic reactor was packed with sand particles (0.5–1 mm), whereas the autotrophic reactor was packed with the elemental sulphur granules. Heterotrophic and autotrophic reactors were inoculated with sludge obtained from denitrifying heterotrophic and autotrophic reactors, respectively, and operated at 28–30 °C in a temperature-controlled room. This temperature range was reported as the optimum range for perchlorate reduction (Ghosh et al., 2011). Dissolved oxygen in the feed containers was stripped using N_2 gas. Feed containers were kept at 4 °C to prevent possible microbial growth. The reactors were covered with aluminum foil to prevent any phototrophic growth. The reactors were fed with tap water supplemented with KNO_3, K_2HPO_4 and ClO_4^-. The feed of the heterotrophic reactor was also supplemented with methanol as a carbon source. The operational conditions of the sequential process is presented in Table 4.1.

Total hydraulic retention time of the sequential process was 2 h (1 h for each). The reactors were sampled at least three times a week and NO_3^--N, NO_2^--N, SO_4^{2-}, pH, dissolved organic carbon, alkalinity, HS^-, ClO_2^-, ClO_3^-, ClO_4^- and CI^- was measured. The influent was also sampled once a week for the measurement of the same parameters except sulphide.

Table 4.1 : Operational condition for sequential process (influent ClO_4^- : 1000 µg/L; HRT: 2 h).

Periods	1	2	3	4
Days	0-21	22 - 37	38 - 56	58 -100
Methanol (mg/L)	61	123	185	277
NO_3^--N (mg/L)	50	75	100	100
NO_3^--N $load_{Auto}$ (mg N/L.d)	600	900	1200	1200
C/N	1.22	1.64	1.85	2.77

4.2.2 Operation of the Reactors

This study was composed of four operational periods (Table 4.1). In the first three periods, nitrate and methanol concentrations were increased gradually from 50 to 100 mg NO_3^--N/L and 61 to 185 mg/L, respectively. In the last period, only the methanol concentration was increased to its maximum level of 277 mg/L. The feed of the heterotrophic bioreactor was supplemented with increasing methanol concentrations corresponding to CH_3OH/NO_3^--N ratio of 1.24, 1.64, 1.85 and 2.77 for the first, second, third and fourth periods, respectively. Although, theoretically 2.47 mg CH_3OH is required for denitrifying each mg NO_3^--N according to Equation (4.1), generally more methanol required. The methanol requirement for heterotrophic denitrification was reported to be 2.65 ± 0.3 (Sahinkaya & Kilic, 2014) and 2.72 mg methanol/mg NO_3^--N (Sahinkaya, Dursun, et al., 2011). In the present study, the reactor was operated under methanol-limiting conditions as the methanol/NO_3^--N ratio in the influent was under 2.47. This was done so as to not contaminate the treated effluent with the unused organic matter. To further investigate the effect of methanol concentration on sulphate and alkalinity, excess methanol was added in the last period. Perchlorate concentration in the feed was kept at 1000 µg/L throughout the process operation. Column reactors were operated for around 100 days under four different operational conditions (Table 4.1).

4.2.3 Analytical Methods

Chlorate, chlorite, chloride, nitrate, nitrite and sulphate 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). Perchlorate was measured by DIONEX 500 system (suppressed conductivity) with the Ion-Pac AS20 column. Alkalinity was measured according to Standard Methods (APHA, 2005). Sulphide was measured spectrophotometrically at 480 nm using a Shimadzu UV–VIS spectrophotometer following the method described by Cord-Ruwisch (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 ions and sulphide measurements.

4.2.4 Batch Adsorption Experiments

Batch adsorption experiments were conducted to identify NO_3^- and ClO_4^- removal mechanisms. In the experiments, 100 mL serum bottles were supplemented with 25 mg NO_3^--N/L and 1000 μg ClO_4^-/L. For the autotrophic experiments, serum bottles were supplemented with 1.5 g elemental sulphur. Similarly, for the heterotrophic experiments, serum bottles were supplemented with 1 g sand and 100 mg/L CH_3OH. The serum bottles were not supplemented with biomass to determine the adsorptive removal of perchlorate, if any. All serum bottles were operated in a temperature-controlled room at 30°C and regularly sampled for the measurements of NO_3^--N and perchlorate.

4.3 Results and Discussions

4.3.1 Effect of nitrate on perchlorate reduction

Perchlorate concentration in groundwater may reach up to 1000 μg/L (Ye et al., 2012). This concentration established as the contamination level in our study. Perchlorate reduction efficiency was investigated under different nitrate loading conditions, keeping the HRT constant while varying the influent nitrate concentration. This was done because nitrate concentration in ground water was reported to vary widely (Liu et al., 2009; Yesilnacar et al., 2008; Ziv-El and Rittmann, 2009) and because there seems to be an important effect of increasing

nitrate concentration on perchlorate reduction efficiency. It is possible that these compounds may compete for the same electron donor.

The influent nitrate concentration was 50 mg NO_3^--N/L in the first period (Figure 4.2). The average effluent perchlorate concentration of the heterotrophic reactor in this period was 734±157 μg/L, only 26% of the perchlorate was removed. Perchlorate concentration further decreased to 616±227 μg/L in the following autotrophic reactor, corresponding to perchlorate reduction rate of 4630 μg/(L.d) for the whole system. Perchlorate was not reduced completely in the whole system suggesting that nitrate inhibited perchlorate reduction and that nitrate and perchlorate compete for the same electron donor. This competitive inhibitory effect is reported for both autotrophic (London et al., 2011) and heterotrophic processes (J Chung, Shin, & Oh, 2010). London et al. reported that 90 and 10 h retention times are required for perchlorate reduction in the presence and absence of nitrate, respectively (London et al., 2011). Another possible reason for not obtaining complete perchlorate reduction is the lengthier requisite acclimation time for reducing low concentrations of perchlorate in the presence of high concentrations of nitrate. Between days 16 and 21, the effluent NO_3^--N concentration was below 1 mg/L in the autotrophic effluent and thus the influent nitrate concentration was increased to 75 mg/L in the second period (Figure 4.2). Although nitrate was reported to inhibit perchlorate reducers, (Choi & Silverstein, 2008), the effluent perchlorate concentrations were decreased in this period. Perchlorate concentrations in the effluents of the heterotrophic and autotrophic reactors were 244±281 and 148±155 μg/L, respectively, an overall reduction efficiency of 85%. The increase in perchlorate reduction efficiency is likely because of the acclimation of perchlorate reducers in both reactors. The increase in the C/N ratio from 1.22 in the first period to 1.64 in the second period may be another reason for higher perchlorate reduction as increasing the amount of electron donor may increase the electron availability for perchlorate reduction.

In the third period, nitrate and methanol concentrations in the feed were further increased to 100 mg NO_3^--N/L and 185 mg/L methanol, a C/N ratio of 1.85. Increased electron donor availability (due to the higher C/N ratio) and bacterial acclimation resulted in high perchlorate reduction rates (Figure 4.2). Average effluent perchlorate concentrations from the heterotrophic and autotrophic reactors

were 146±164 μg/L and undetectable (<4 μg/L), respectively, corresponding to perchlorate reduction rates of 20.5 and 3.5 mg/(L.d). In the last period, the influent nitrate concentration was kept at 100 mg/L, however the methanol concentration was increased. Increasing the methanol concentration resulted in a decrease in perchlorate reduction efficiency in the heterotrophic reactor. Adding excess methanol to the reactor stimulated excess bacterial growth and thus affected the reactor hydraulics. Regardless, the subsequent autotrophic reactor further decreased perchlorate concentration.

Perchlorate reductions of as much as 250 mg/L of the initial concentration has been reported for heterotrophic processes (Fox et al., 2014). On the other hand, autotrophic processes were also reported to be effective for both high (5–8 mg/L) and low (60–120 μg/L) perchlorate concentrations (Sahu et al., 2009). However, according to the best of our knowledge, this is the first study on nitrate and perchlorate reduction using sequential heterotrophic and autotrophic denitrification processes. Currently, there is no standard available for maximum perchlorate concentration from WHO, European Union or US-EPA regulations (Blake et al., 2009). The EPA set a reference dose of 0.7 μg perchlorate/kg body weight corresponding to a drinking water equivalent level of 24.5 μg/L assuming that water is the only source of perchlorate intake (Tikkanen, 2006), although perchlorate may also be present in milk, rice and fish (Srinivasan & Sorial, 2009). The heterotrophic–autotrophic sequential system successfully reduced 100 mg NO_3^--N/L and 1000 μg/L perchlorate and the sequential process has advantages over single-stage heterotrophic or autotrophic processes. High concentrations of nitrate together with perchlorate have also been treated in a hydrogen-based membrane biofilm reactor achieving complete reduction of perchlorate (Zhao et al., 2013).

Despite handling and storage as safety issues; hydrogen is an ideal electron donor since it is non-toxic, relatively inexpensive and sparingly soluble (Robert Nerenberg et al., 2002). Fe^0 has been successfully used in permeable reactive barriers for the remediation of chlorinated volatile organic compounds (Lai, Lo, Birkelund, & Kjeldsen, 2006; Richardson & Nicklow, 2002) and could be an alternative for ClO_4^- reduction. Fe^{2+} and S^{2-} are examples of other inorganic electron donors used by ClO_4^- reducing bacteria (Weber, Achenbach, & Coates, 2006).

According to Equation (4.2), the sulphur-based autotrophic reduction of each gram of ClO_4^- generates 2.8 grams of sulphate. Similarly, the autotrophic reduction of each gram of NO_3^--N generates 7.56 grams of sulphate (Equation (4.3)). During the autotrophic reactor operation, sulphate was only calculated according to the nitrate concentration in the influent, as there were orders of magnitude less perchlorate. Low biomass production from the autotrophic denitrification process is advantageous as it leads to low sludge production and decreases the probability of column clogging. Although bioreactors treating only perchlorate have also been reported (Son et al., 2006), long acclimation periods are required at low perchlorate concentrations because of low biomass yield (Sahu et al., 2009). Nerenberg et al. suggested a minimum perchlorate concentration of 0.14 μg/L to support bacterial growth when perchlorate is the primary electron acceptor (Robert Nerenberg et al., 2006).

The energy gained by the reduction of NO_3^- or ClO_4^- is very similar (−91 and −113 kJ/e^-, respectively). Other factors, such as, concentration, biomass type, reactor operating conditions may determine the outcome of nitrate/perchlorate competition. Some researchers reported that perchlorate reduction is initiated simultaneously with nitrate; however, its complete reduction was inhibited by nitrate (Ghosh et al., 2011). Choi and Silverstein reported that when acetate was reduced 50% to well below the stoichiometric requirement, perchlorate reduction decreased by 70%, while denitrification decreased by only 20% (Choi & Silverstein, 2008). A combination methanol-based heterotrophic and sulphur-based autotrophic denitrification process is advantageous and results in less organic contamination risk as well as low sulphate and acidity generation. The heterotrophic–autotrophic sequential process could reduce high concentrations of nitrate and perchlorate simultaneously without releasing other contaminants to effluent.

4.3.2 Nitrate reduction efficiency in the presence of perchlorate

Throughout the study, the initial nitrate concentration of 50 mg/L was increased to 75 and 100 mg NO_3^--N/L while perchlorate concentration was kept at 1000 μg/L. During the first period, influent nitrate was 50 mg NO_3^--N/L and average concentration in the heterotrophic effluent was 26.86±8.76 mg NO_3^--N/L corresponding to 48% nitrate removal by the heterotrophic process. Methanol concentration in the feed was around 61 mg/L, theoretically enough to reduce 25 mg

NO_3^--N/L. According to these reduction rates, 2.59 grams of methanol were used to reduce each grams of NO_3^--N. Similar methanol/NO_3^--N ratios were found in other studies (2.65 ± 0.3 in (Sahinkaya & Kilic, 2014) and 2.72 in (Sahinkaya, Dursun, et al., 2011)). The effluent nitrate concentration in the first period was reduced to low levels by the autotrophic reactor. Average effluent nitrate concentration from the autotrophic process was 6.90 ± 8.75 mg NO_3^--N/L in the first period. Therefore, with the sequential heterotrophic–autotrophic processes high denitrification efficiencies can be achieved.

In the second stage, NO_3^--N and methanol concentrations were increased to 75 and 123 mg/L, respectively (Figure 4.2). The effluent nitrate concentration from the heterotrophic reactor during this period averaged 17.36±5.70 mg NO_3^--N/L, corresponding to 57.64 mg/L NO_3^--N heterotrophically reduced. Theoretically, 120 mg/L methanol is required to reduce 48.5 mg/L NO_3^--N (Equation (4.1)). The theoretical nitrate removal by the heterotrophic process was in good agreement with the measured concentrations. Nitrate concentration was further reduced in the autotrophic reactor to 0.5 mg NO_3^-/L.

In the third period, nitrate and methanol concentrations were increased to 100 and 185 mg/L, respectively, and the average effluent nitrate concentrations for the heterotrophic and autotrophic reactors were 20.2 ± 4.9 and 1.32 ± 3.84 mg NO_3^--N/L, respectively. In all cases, the aim was to reduce a portion of nitrate in the heterotrophic process and the rest in the autotrophic process eliminating the organic and nitrate/nitrite contamination of treated effluent. Autotrophic reduction of nitrate with concentrations above 25 mg NO_3^--N/L may result in excess sulphate in the effluent (Equation (4.3)). Therefore, organic supplementation to the heterotrophic reactor was controlled so as not to exceed 25 mg NO_3^--N/L in the influent of the autotrophic process.

In order to monitor the effect of methanol on effluent sulphate concentration, methanol in the feed was increased to its maximum level of 275 mg/L in the last period. This high methanol concentration led to an almost complete reduction of 100 mg NO_3^--N/L in the heterotrophic reactor and therefore low concentrations of nitrate remained for the autotrophic process. As a result, sulphate concentrations for the last period decreased. The average nitrate concentration for the heterotrophic and

autotrophic processes in this period were 4.21 ± 7.62 and 0.02 ± 0.05 mg NO_3^--N/L, respectively

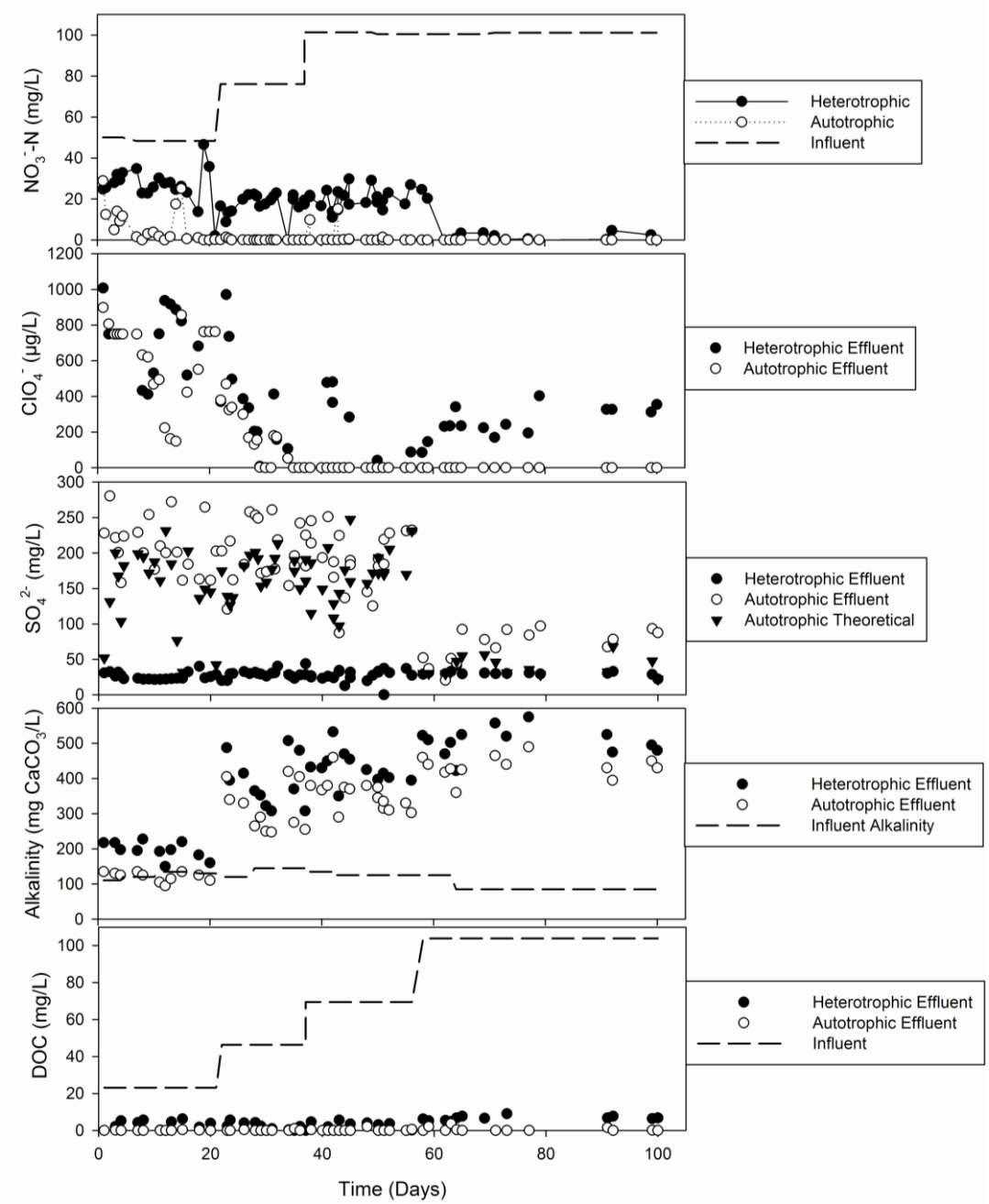

Figure 4.2 : Feed and effluent NO_3^--N, sulfate, alkalinity and DOC variations of both reactors. Influent ClO_4^- concentration was 1000 µg/L. Theoretical sulfate concentration for the autotrophic reactor was calculated according to Equation 4.3.

Although nitrite was observed in the effluent of both reactors in the first 20 days of operation, later it was only detected in the effluent of the heterotrophic reactor, probably because, as reported by other researchers, the electron donor was exhausted

(Chiu & Chung, 2003). Hence, the autotrophic reactor behaved as a polishing step after the heterotrophic bioreactor. A poor C/N ratio leads to improper denitrification and the average nitrite concentration in the effluent of the heterotrophic reactor was 3.79 ± 5.45 mg NO_2^--N/L throughout the study. Batch adsorption studies revealed that ClO_4^- and NO_3^- were not adsorbed on elemental sulphur/sand for both reactors (data not shown).

4.3.3 Sulphate production

The measured sulphate concentrations and the theoretical effluent sulphate concentrations are presented in Figure 4.2. In the first three periods, the heterotrophic reactor was adjusted to reduce a certain portion of influent nitrate. According to Equation (4.3), each mg NO_3^--N produces 7.56 mg SO_4^{2-} by autotrophic denitrification. In order to control the effluent sulphate concentration, 25 mg NO_3^--N was allocated for the autotrophic process and methanol was added into the heterotrophic reactor accordingly. In the first period, the addition of 61 mg methanol resulted in the heterotrophic denitrification of 24 mg NO_3^--N/L (Figure 4.2) Hence, 26±8.76 mg NO_3^--N/L remained in the influent of the autotrophic reactor. However, the nitrate in the influent of the autotrophic reactor was not completely reduced and remained in the effluent with an average value of 6.9±8.75. The sulphate concentration in the effluent of the autotrophic rector originated from the autotrophic reduction of 19.1 mg/L nitrate and the influent sulphate concentration of 26±5 mg/L. The average effluent sulphate concentration of the autotrophic reactor during the first period was 210±37 mg SO_4^{2-}/L, which was much higher than theoretically calculated (170 mg/L) (Figure 4.2). It is possible that oxygen may leaked into the system during sampling or reactor operation to increase effluent sulphate concentration in the first period. Although it is also possible that a disproportionate amount of S^0 could have caused the increased SO_4^{2-} production, the absence of sulphide in the reactor eliminated this possibility (Ju et al., 2007). However, the effluent sulphate concentration in this period was still below the regulations (250 mg/L) of TS266 and the US-EPA.

In the second period, influent NO_3^--N and methanol concentrations proportionally increased and thus resulted in an increase in the heterotrophic denitrification. Average nitrate concentrations in the influent of the autotrophic reactor was around

17 mg NO_3^--N/L (Figure 4.2), theoretically producing 129 mg/L sulphate in the case of complete autotrophic denitrification. Average effluent sulphate concentration in this period was 200±40.20 mg SO_4^{2-}/L. In the third period, the influent nitrate concentration was increased to its maximum level throughout the study; however, the ratio between nitrate and methanol was kept constant. Hence, effluent sulphate concentrations were not changed significantly and averaged 193±42 mg SO_4^{2-}/L, and a significant amount of the nitrate was heterotrophically reduced (Figure 4.2).

In the last period, in order to decrease the effluent sulphate concentration, excess methanol was added to the feed. Hence, the nitrate concentration in the heterotrophic effluent was significantly decreased. The average nitrate concentration in the effluent of the heterotrophic reactor was 4.21±7.62 mg NO_3^--N/L. In this period, lower nitrate concentrations were reduced in the autotrophic reactor and therefore a lower sulphate concentration were produced. The average effluent sulphate concentration of the autotrophic reactor was 69.40±23.60 mg/L (Figure 4.2). Addition of organic electron donors reduced effluent sulphate concentrations. In another study, Sahinkaya and Dursun decreased the sulphate concentration to below the regulatory level in a sulphur-based autotrophic denitrification process by supplementing methanol to the reactor, which stimulated simultaneous autotrophic and heterotrophic denitrification (the mixotrophic denitrification process) (Sahinkaya & Dursun, 2012).

In a heterotrophic–autotrophic sequential denitrification process, the effluent sulphate concentration can be controlled by adjusting the dose of organic carbon in the heterotrophic process. Similar reactions in mixotrophic reactors have also been reported (Sahinkaya & Dursun, 2012). Throughout the study, thiosulphate was not detected in the effluent of the bioreactor, which means sulphur was completely oxidized to sulphate.

4.3.4 Variations of process alkalinity

Increasing the nitrate and methanol concentrations also increased alkalinity production in the bioreactor due to the alkalinity generation during the heterotrophic process according to Equation (4.1). According to Equation (4.3), 1 mol nitrate removed under autotrophic conditions produces 1.28 mol hydrogen ions, corresponding to 4.57 grams $CaCO_3$ consumed per gram of nitrate removed. On the

other hand, according to Equation (4.1), 3.57 grams of alkalinity is produced per gram of NO_3^--N denitrified heterotrophically.

In the first period, the influent methanol and nitrate concentrations were 61 and 50 mg NO_3^--N/L, respectively. In this period, the heterotrophic and autotrophic effluent alkalinity concentrations were 196±24.70 and 121.4±13.43 mg $CaCO_3$/L. The heterotrophic denitrification was not completed in the first period and nitrite accumulated in the system. The average alkalinities for the influent and effluent of the heterotrophic reactor were 124±10 and 196±25 mg/L, respectively. In the autotrophic reactor, around 75 mg $CaCO_3$/L was consumed corresponding to 17 mg NO_3^--N/L reduced by the autotrophic reactor. Measured autotrophic reactor influent and effluent nitrate concentrations were 26.80 ± 8.76 and 6.9 ± 8.75 mg NO_3^--N/L, respectively, which shows that the alkalinity consumption in the autotrophic reactor completely fits with theoretically calculated levels.

In the second period, influent nitrate and methanol concentrations were increased to 75 and 123 mg/L, respectively, and the heterotrophic reactor alkalinity increased to 392±76 mg/L. The autotrophic reactor consumed around 75 mg $CaCO_3$/L as a result of 16 mg NO_3^--N/L reduction. In the third period, methanol and influent nitrate nitrogen concentrations were further increased to 100 and 185 mg/L. The alkalinity concentrations in the heterotrophic and autotrophic effluent increased to 429±45.55 and 354±42.25 mg $CaCO_3$/L, respectively.

In the last period, the methanol concentration increased to its maximum level of 277 mg/L to increase heterotrophically reduced nitrate. In this period, heterotrophic and autotrophic effluent alkalinity concentrations increased to 506±40 and 433±32 mg $CaCO_3$/L, respectively, and 96% of the nitrate was reduced in the heterotrophic reactor. Combining heterotrophic and autotrophic processes eliminated alkalinity requirement of the autotrophic process and decreased the organic carbon requirement of the heterotrophic process. Lee et al. reported that when methanol or acetate was used as a carbon source, complete denitrification without alkalinity addition was observed when 60% and 44%, respectively, of nitrate was heterotrophically denitrified. When glucose was used as a carbon source, 70% of the nitrate is used by heterotrophs to balance the alkalinity (D. U. Lee, Lee, Choi, & Bae, 2001). Limestone is used to buffer acidity produced in autotrophic denitrification (Sahinkaya, Dursun, et al., 2011). The use of limestone seems to be an effective and

economical way to supplement the alkalinity, but its limited dissolution rate makes it difficult for limestone to provide enough alkalinity at high nitrate ladings (Oh et al., 2001).

When autotrophic denitrification alone was considered, additional alkalinity is required. This alkalinity may be supplied by a support material in the reactor or by a dissolved form such as $NaHCO_3$ in the feed (Sahinkaya & Dursun, 2012). In autotrophic denitrifying bioreactors limestone (Sahinkaya, Dursun, et al., 2011) or oyster shells (Sahu et al., 2009) have been reported to be alkalinity sources.

4.3.5 Residual organics

Methanol was added in all periods and it was almost completely removed in the bioreactor. Influent methanol concentrations were 61, 123, 185 and 277 mg/L for the first, second, third and fourth periods, respectively. In the heterotrophic–autotrophic sequential process, the autotrophic reactor was used to decrease nitrate, nitrite and perchlorate concentrations further. DOC concentration in the effluent of heterotrophic reactor averaged 3.80±2.90 mg/L. The highest effluent DOC concentrations (7.10 ± 1.90) were measured in the last period, probably because of the much higher methanol dosing in this period. The remaining DOC in the effluent of the heterotrophic reactor was also further oxidized in the autotrophic reactor. In this period (fourth period), the average effluent DOC concentration of the autotrophic reactor was 2.65±2.16 mg/L. For the first three periods, the effluent DOC concentrations of the heterotrophic reactor were similar. In the first, second and third periods, effluent DOC concentrations of the heterotrophic process were 3.06±2.34, 1.99±1.90 and 2.22±2.08 mg/L, respectively. DOC concentrations in the autotrophic reactor effluent for these periods were below 0.20 mg/L, illustrating that the autotrophic reactor may also behave as a polishing step not only for nitrate, nitrite and perchlorate, but also for the remaining DOC.

With organic electron donors, nitrate reduction is fast and efficient. However, there are some concerns regarding the utilization of organic carbon sources for drinking water treatment. Toxic by-products may be formed during the disinfection processes. Additionally organic carbon in water distribution systems may promote bacterial growth. Completely heterotrophic systems must deal with the above mentioned issues. Additionally, a poor C/N ratio will lead to improper denitrification,

accumulation of nitrite or extra production of nitrous other than nitrogen gas (Kim et al., 2002). The sequential process may overcome such concerns as the autotrophic reactor could be used as a polishing unit for organic carbon, accumulated nitrite and other unwanted residuals from the heterotrophic process.

4.4 Conclusions

Simultaneous reduction of nitrate (up to 100 mg NO_3^--N/L) and perchlorate (1000 μg/L) was achieved in the sequential heterotrophic–autotrophic denitrifying process. Depending on the methanol concentration in the feed, nitrate was reduced in the heterotrophic reactor to some extent. The nitrate that remained was further reduced in the following autotrophic reactor. Perchlorate reduction was initiated in the heterotrophic part and completed in the autotrophic reactor. While perchlorate removal efficiencies were 38% and 85% for the first and second periods, respectively, complete reduction was attained in the following periods due to increased organic carbon dosing and bacterial acclimation. The effluent perchlorate concentrations were below the EPA's temporary reference dose of 24.5 μg/L. Nitrate and sulphate for all tested periods were under the drinking water guideline value of 10 mg NO_3^--N/L and 250 mg SO_4^{2-}/L. The sequential process allowed control of effluent sulphate concentration, eliminating the alkalinity need of autotrophs and maintaining a low effluent organic concentration.

5. SIMULTANEOUS NITRATE AND PERCHLORATE REDUCTION IN ELEMENTAL SULFUR-BASED AUTOTROPHIC AND HETEROTROPHIC PROCESSES

5.1 Introduction

Perchlorate is a soluble anion that consists of a chlorine atom at the center surrounded by four oxygen atoms in a tetrahedral array. It has been used in rocket propellants, highway safety flares, air-bag inflators, fireworks and matches (Motzer, 2001). Extensive perchlorate contamination in water sources was detected in 1997, with the development of instrumental techniques that used highly sensitive chromatography. These techniques allowed detection of perchlorate at levels of less than 4 μg/L. The investigations demonstrated that the real extent of perchlorate contamination had been underestimated (Collette et al., 2003; Motzer, 2001). Other than in water sources, perchlorate has been detected in rice, fish and dairy products, vegetables and even breast milk (Srinivasan & Sorial, 2009). Trace quantities of perchlorate have also been measured in rainwater (Dasgupta et al., 2005).

Perchlorate blocks iodine uptake by the thyroid, decreasing thyroid hormone (triiodothyronine-T3 and thyroxine-T4) concentrations called hypotyreosis (Greer et al., 2002). This depletion can trigger many diseases related to the basal metabolism. Currently, there is no standard for maximum perchlorate concentration set by WHO, EU or US-EPA regulations (Blake et al., 2009). Only EPA sets a reference dose of 0.7 μg perchlorate/kg body weight. This corresponds to a drinking water equivalent level of 24.5 μg/L assuming water is the only source of perchlorate consumption (Tikkanen, 2006). Some states in the USA have established an advisory level for perchlorate: in California it is 6 μg/L, Masachusetts, 2 μg/L, and Texas 4 μg/L (Srinivasan & Sorial, 2009).

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Nitrate is a common co-contaminant of perchlorate and many studies focus on the treatment of both anions (Burge & Halden, 1999; Jinwook Chung, Nerenberg, et al., 2007; Ghosh et al., 2011; Van Ginkel et al., 2008). One reason that nitrate appears alongside perchlorate is that the nitrate deposits in Chile also contain perchlorate. These deposits have been used as fertilizers in the Unites States for over a century (Srinivasan & Sorial, 2009). Nitrate can cause methemoglobinemia when ingested by infants, and could cause carcinoma, malformation and mutation when transformed into nitrosoamines (Della Rocca et al., 2007). Some wells in Harran Plain, in the Sanliurfa region of Turkey, contain nitrate as high as 180 mg NO_3^--N/L. The average concentration for the whole plain is 35 mg NO_3^--N/L (Yesilnacar et al., 2008). The US-EPA set the maximum contaminant levels at 10 mg/L NO_3^--N and 1.0 mg/L NO_2^--N for drinking water (K. C. Lee & Rittmann, 2002). Biological reduction is the most commonly used method for detoxification of these oxyanions, due to the fast reaction rate and the elimination of expensive catalysts or chemicals required (Srinivasan & Sorial, 2009). Biological reduction of nitrate and ClO_4^- can be achieved using organic (Sahinkaya & Dursun, 2012; Wang et al., 2012) or inorganic electron sources (Ju et al., 2008). Expected stoichiometry of the reactions for both electron donors is presented in Equation 5.1 and 5.2.

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (5.1)$$

$$55S^0 + 50NO_3^- + 38H_2O + 20CO_2 + 4NH_4^+ \rightarrow 4C_5H_7O_2N + 55SO_4^{2-} + 25N_2 + 64H^+ \quad (5.2)$$

Heterotrophic reduction of oxyanions is faster and more effective compared to autotrophic processes. However, 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).

To overcome these problems, one can use autotrophic perchlorate-reducing bioreactors, in which inorganic electron donors such as H_2 (Ziv-El & Rittmann, 2009), Fe(0) (Cao et al., 2005) and elemental sulfur (Ju et al., 2008) have also been added as electron donors. Cao et al. (2005) examined the perchlorate reduction process 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 this reaction is favorable in terms of thermodynamics (activation energy was calculated as 79.02 ±7.75 kJ $mole^{-1}$), the authors noted that 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 mM d^{-1} with S^0 and aerobic process sludge for the electron donor and inoculum respectively (Ju et al., 2008). Reduction rates of Hydrogen and Fe^0 with the same inoculum were ≥0.37 mM d^{-1} on day 8 and 0.085 mM d^{-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., 2013, 2015). Granular S^0 provides for a slow release of electrons on demand, advantageously eliminating dose adjustments in a simple and reliable operation. On the other hand, acidity and sulfate production are the drawbacks of the autotrophic system. Therefore studies are needed to understand the process performance under varying operational conditions, and to provide some operational data before its use in continuous scale applications. Our current study examines the simultaneous reduction of nitrate and perchlorate in column reactors. Currently two autotrophic and one heterotrophic denitrifying reactor are in operation. To stimulate perchlorate reduction in these reactors, batch tests are needed to identify possible toxic effects and the determination of startup concentration of perchlorate for continuous system. Hence, this study aims to discover process responses for simultaneous nitrate and perchlorate reduction under varying perchlorate loads. The presence of possible perchlorate inhibition to denitrification process was investigated.

5.2 Materials and Methods

5.2.1 Denitrifying Batch Bioreactors

Three set denitrifying batch bioreactors were operated in parallel. Elemental sulfur and methanol were used as electron sources in Reactor 1-3 and Reactor 2, respectively. Each set consists of six individual reactors with different concentrations of perchlorate. The working volume of the reactors was 100 mL. Reactors utilized in the first set were packed with 1.5 g elemental sulfur granules (0.5–1 mm). The second set of reactors contained sand (1 g) and the third set had 1.5 g elemental sulfur and 1 g limestone (1-2 mm) particles. The reactors were inoculated with a

sludge obtained from autotrophic and heterotrophic denitrifying reactors and operated at 28–30 ºC, which is in the optimum range for perchlorate reduction (Ghosh et al., 2011). Dissolved oxygen in the feed was stripped using N_2 gas. The reactors were covered with aluminum foil to prevent any phototrophic growth. The reactors were fed with tap water supplemented with KNO_3, K_2HPO_4 and ClO_4^-. The feed of the second set heterotrophic reactors was also supplemented with methanol as a carbon source. The reactors were sampled several times during the 90h test period.

5.2.2 Reactor Operation

In this study 18 batch reactors were operated in parallel in three set of experiment. All sets contained six reactors with different concentrations of perchlorate. The concentration of perchlorate in the reactors was set at 100, 200, 400, 800 and 1500 µg/L. One control reactor in each set was operated without perchlorate addition. The feed of the heterotrophic bioreactor was supplemented with methanol at a concentration of 100 mg/L, corresponding to C/N ration of 1.5. McAdam and Judd, (2007) reported optimum C/N ratio ranging from 1.45 to 1.52, at which effluent organic, nitrate and nitrite concentrations were low (Ewan J. McAdam & Judd, 2007). Nitrate, nitrite, sulfate and chloride concerntrations were measured by analyzing suppressed conductivity ion chromatography using the Shimadzu HIC-SP system fitted with a DIONEX Ion-Pac AS9-HC column (4 mm x 250 mm). Perchlorate was measured by DIONEX 500 system (suppressed conductivity) with Ion-Pac AS20 column (2 mm x 250 mm).

5.2.3 Batch Adsorption Experiments

Batch adsorption experiments were conducted to identify NO_3^- and ClO_4^- removal mechanisms. In the experiments, 100 mL serum bottles were supplemented with 25 mg/L NO_3^--N and 1000 µg/L ClO_4^-. For the autotrophic denitrification experiments, serum bottles were supplemented with 1.5 g elemental sulfur and 1 g limestone. Similarly, for the heterotrophic denitrification experiments, serum bottles were supplemented with 1 g sand and 100 mg/L CH_3OH. All serum bottles were operated in a temperature controlled room at 30 ^oC and regularly sampled for the measurements of NO_3^--N and perchlorate.

5.3 Results

5.3.1 Perchlorate and Nitrate Removal Performances of the Autotrophic Reactor (Elemental Sulfur as Electron Source and $NaHCO_3$ as Alkalinity in the Feed)

The reduction of 25 mg NO_3^--N/L was completed in 50 h in reactor 1. The NO_3^--N concentration in the flask containing no perchlorate was 1.47 mg/L at the end of 35 h corresponding to 94% denitrification efficiency. At this point, effluent NO_3^--N concentrations for perchlorate-containing bottles were similar to that obtained in the bottle containing no perchlorate (varied between 0.55 – 5.20 regardless of influent perchlorate). It could be noted that the denitrification performance was not affected by influent perchlorate concentration. This situation may be explained by the bacterial preference of electron acceptors. Ziv-El and Rittmann investigated a hydrogen-based membrane biofilm reactor to investigate the reduction of various oxyanions. The reduction order was oxygen, nitrate, nitrite and then perchlorate, when the electron donor was limited (Ziv-El & Rittmann, 2009). Accordingly in our study, perchlorate reduction (discussed later) was completed after the complete reduction of nitrate. In our process elemental sulfur is the sole electron source and its solubility is quite low: only 5 μg/L at 20^oC (Ju et al., 2007). Although the reactor was operated in sulfur-abundant conditions, the dissolved sulfur was probably first utilized for denitrification. Effluent nitrate concentrations were under detectable level for all reactors after 50h.

Tap water was used to prepare synthetic drinking water samples and the average sulfate concentration of tap water was 30±10.60 mg/L. According to Equation 5.2, 7.56 g sulfate is produced for each g NO_3^--N reduced. In the end of test, 25 mg NO_3^--N was completely reduced in all bottles and varying sulfate concentrations (237-273 mg/L) were measured. Theoretically 25 mg NO_3^--N, together with the sulfate in the tap water, would produce 219±10.60 mg SO_4^{2-}. Sulfate concentrations were higher than theoretically calculated. High effluent sulfate concentrations are likely due to the leakage of oxygen during sampling or feeding, although some precautions (e.g. bubbling nitrogen gas through feed after preparation and keeping the feed in a collapsible container) were taken.

Perchlorate concentrations for each sampled time period is shown in Figure 5.1. Influent perchlorate concentrations varied between 100 and 1500 µg/L. Perchlorate was detected in the effluent at 72h for all bottles but complete reduction was observed in the bottles containing 100, 200 and 400 µg/L at 90h.

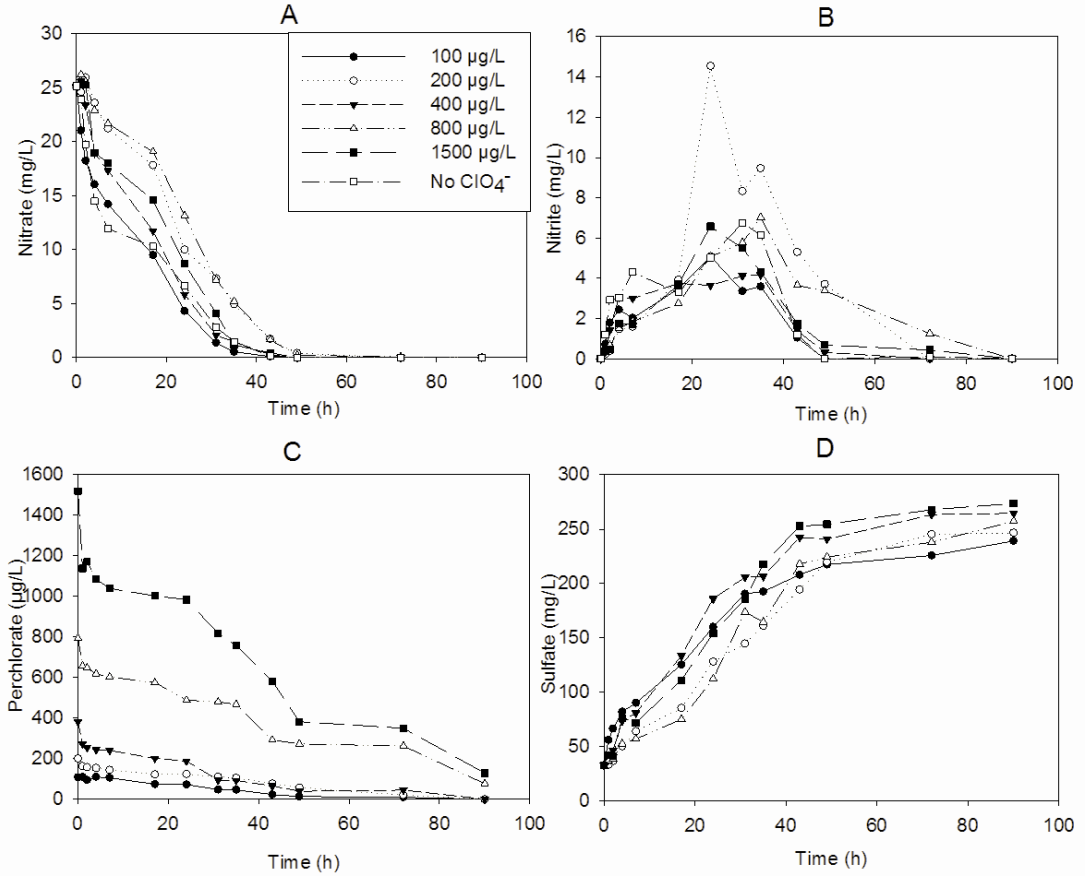

Figure 5.1 : Performances of autotrophic reactors fed with elemental sulphur and $NaHCO_3$ (A: Nitrate; B: Nitrite; C: Perchlroate and D: Sulfate concentrations over time).

As indicated in similar studies perchlorate degradation was initiated quickly without any lag phase, but its complete reduction was achieved only after complete nitrate and nitrite reduction. As mentioned before, nitrate was completely reduced in 49h, however perchlorate reduction was not completed at this time. Ghosh et al. (2011) reported similar results; they observed that perchlorate reduction started quickly but the process was inhibited by the presence of nitrate and nitrite (Ghosh et al., 2011). Quick reduction without lag phase is shown in Figure 5.1C for bottle containing 1500 µg/L perchlorate. In this bottle, around 30% perchlorate had been eliminated by 1h, but the remaining 62% was reduced over the following 90h period. The effect of nitrate on perchlorate degradation by denitrifying perchlorate reducers has been

reported by several studies (Bruce et al., 1999). Suppression of the perchlorate reductase enzyme by nitrate as suggested previously (Batista & Liu, 2001) might be a reason for this — a hypothesis that needs further molecular investigation.

Little reduction of perchlorate was observed in the bottles containing only elemental sulfur and elemental sulfur/limestone mixture. On the other hand, continuous decrease of perchlorate concentration was observed in the bottles containing elemental sulfur and inoculates. This data suggests that elemental sulfur provides the necessary electrons. In the literature, studies on perchlorate reduction focused on the perchlorate reduction/chloride recovery to confirm perchlorate was reduced to chloride. However, this observation is problematic in the treatment of drinking water especially groundwater. Tap water was used in our tests and initial chloride concentration was around 20 mg/L. Up to 300 mg/L chloride concentrations was measured in the groundwater samples of Harran Plain located in South East Turkey. Therefore observation of molecular transformation of ClO_4^- to Cl^- is difficult when observing an increase in µg/L range over high mg/L Cl^-.

5.3.2 Perchlorate and Nitrate Removal Performances of the Heterotrophic Reactor (Methanol as Electron Source)

Methanol was utilized in the heterotrophic reactor, and faster reaction kinetics were observed in this reactor. Denitrification of 25 mg NO_3^--N/L was completed in around 17 h. In the test bottle where no perchlorate was added, effluent NO_3^--N/L concentration was 0.59 mg/L corresponding to a 98% reduction efficiency over the 7h period. However at this time, 100 – 1500 µg ClO_4^-/L perchlorate containing flasks exhibited denitrification performances varying between 80-90%. Nitrate reduction was complete in all bottles except the bottle containing 1500 µg ClO_4^-/L in 17h; nitrate dropped below detectable levels in this bottle at 24h. Utilization of an organic electron donor resulted in faster reaction rates. Sahinkaya and Kilic found 150 mg/(L.d) denitrification rate for methanol based denitrification whereas the same ratio for an elemental sulfur-based system was 102 zozik rate (Sahinkaya & Kilic, 2014). The most common carbon sources are ethanol, methanol and acetic acid, which have been used in denitrification processes in full scale plants for drinking water treatment (Matějů et al., 1992). However, there is some concern 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 from organic carbon sources build up during the disinfection processes. While completely heterotrophic systems have such concerns, a poor C/N ratio leads to improper denitrification, while a high C/N ratio may cause accumulation of organic contaminants (Kim et al., 2002). A combination of an elemental sulfur-based autotrophic process with a methanol-based heterotrophic process could overcome all of these drawbacks. Proper dosing of methanol to autotrophic denitrifying reactor could completely remove nitrate and perchlorate without excess sulfate or DOC contamination in the effluent.

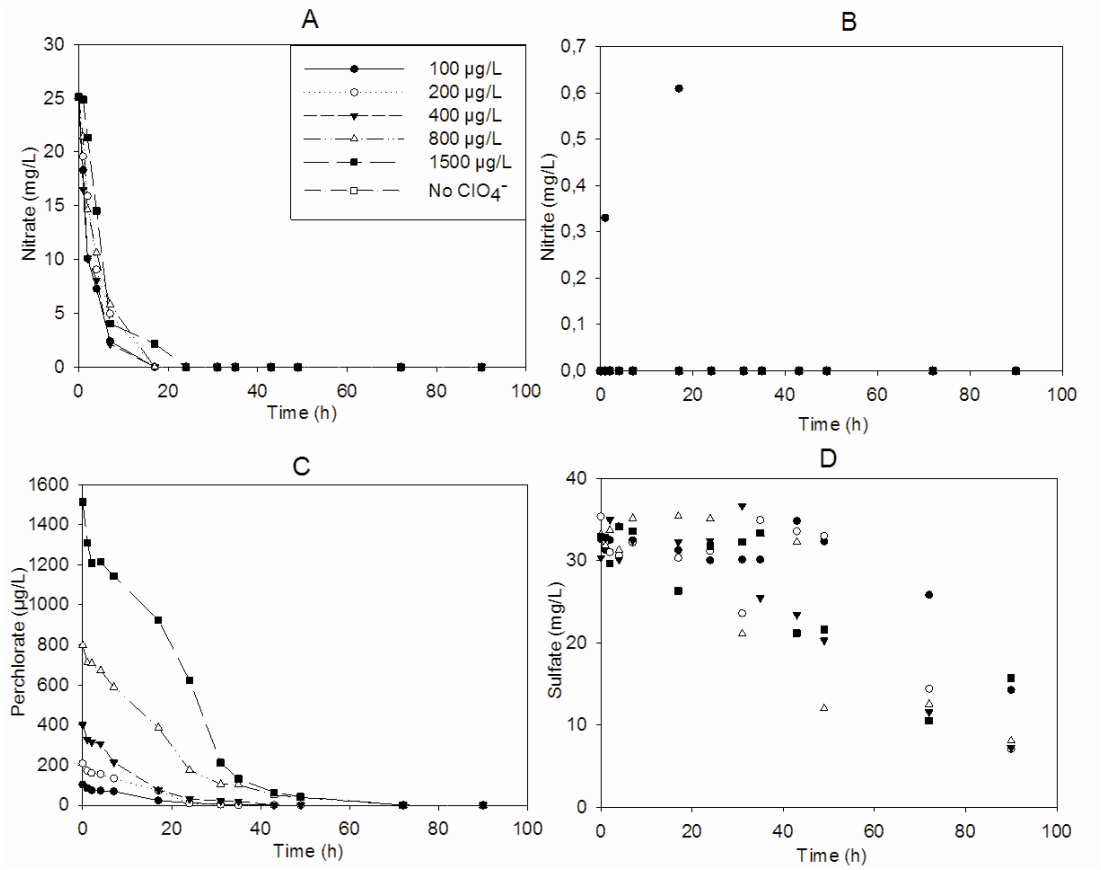

Figure 5.2 : Performances of heterotrophic reactors fed with methanol (A: Nitrate; B: Nitrite; C: Perchlorate and D: Sulfate concentrations over time).

As can be seen in the Figure 5.2, at the end of 43h bottles containing 100, 200, and 400 µg/L perchlorate were completely reduced and 50.48 and 63.16 µg/L perchlorate was measured in the bottle containing 800 and 1500 µg/L perchlorate respectively. As can be seen in the bottles containing 800 and 1500 µg/L perchlorate, the reduction initiated quickly. However perchlorate degradation was completed after complete nitrate reduction. Almost complete perchlorate removal was observed at 49h. When

compared with R1 methanol accelerated both the nitrate and perchlorate reduction process. Acetate as another organic donor is the most commonly used electron donor in the biological reduction of perchlorate (Bardiya & Bae, 2011). Wang et al. (2013) compared the reduction performances of hydrogen and acetate. They found that 15 and 8 days were required when hydrogen and acetate was used respectively, for the reduction of 2.5 mg/L perchlorate (Wang et al., 2013).

As shown in Figure 5.2, nitrate removal was achieved by 24h. After complete nitrate reduction, a sharp decrease in perchlorate was observed in the bottles containing 1500 µg/L perchlorate. Although perchlorate reduction was inhibited in the presence of nitrate, its reduction accelerates immediately after complete nitrate reduction. Authors studied with both organic (Wang et al., 2012) or inorganic (London et al., 2011) electron donors reported this inhibitory effect of nitrate on perchlorate. London et al. (2011) reported that a 90 h retention time is required when nitrate is present whereas 10 h is expected to reduce perchlorate in the absence of nitrate (London et al., 2011). Despite its inhibitory effects, nitrate may be required for perchlorate reduction as low perchlorate concentrations may not sustain bacterial growth and nitrate may be required as the primary electron acceptor. Nerenberg et al., (2006) determined the minimum perchlorate concentration to support bacterial growth to be 14 µg/L(Robert Nerenberg et al., 2006).

5.3.3 Perchlorate and Nitrate Removal Performances of the Autotrophic Reactor (Elemental Sulfur as Electron Source and Limestone as Alkalinity in the Reactor)

Denitrification performance of R3 is shown in Figure 5.3, Complete nitrate reduction was achieved in the bottle containing 100 µg/L perchlorate at 49h, however the nitrate reduction efficiency of the other bottles varied between 44-92% at this time. At the end of 90h period, some of the bottles still contained nitrate. To investigate the possible effect of bacteria concentration on the denitrification process, VSS analysis was conducted at the end of the study. This analysis found that the denitrification performance was independent from bacteria concentration in the bottles (data not shown). The only difference between R3 and R1 was the alkalinity source. In R3, limestone was used whereas dissolved $NaHCO_3$ was used in R1. Limestone-fed denitrification bottles showed a slower denitrification rate than that obtained in R1.

Effluent sulfate concentration was not affected by influent perchlorate concentrations (Figure 5.3). A slower denitrification process resulted in lower sulfate concentrations than that obtained in R1. As can be seen in Figure 5.3, higher sulfate concentration was obtained in two of the six batches, probably showing the presence of oxygen in the reactor. The average effluent sulfate concentration (238.9 ±26.41 mg/L) was below the US-EPA, EU, and Turkish drinking water standard of 250 mg/L.

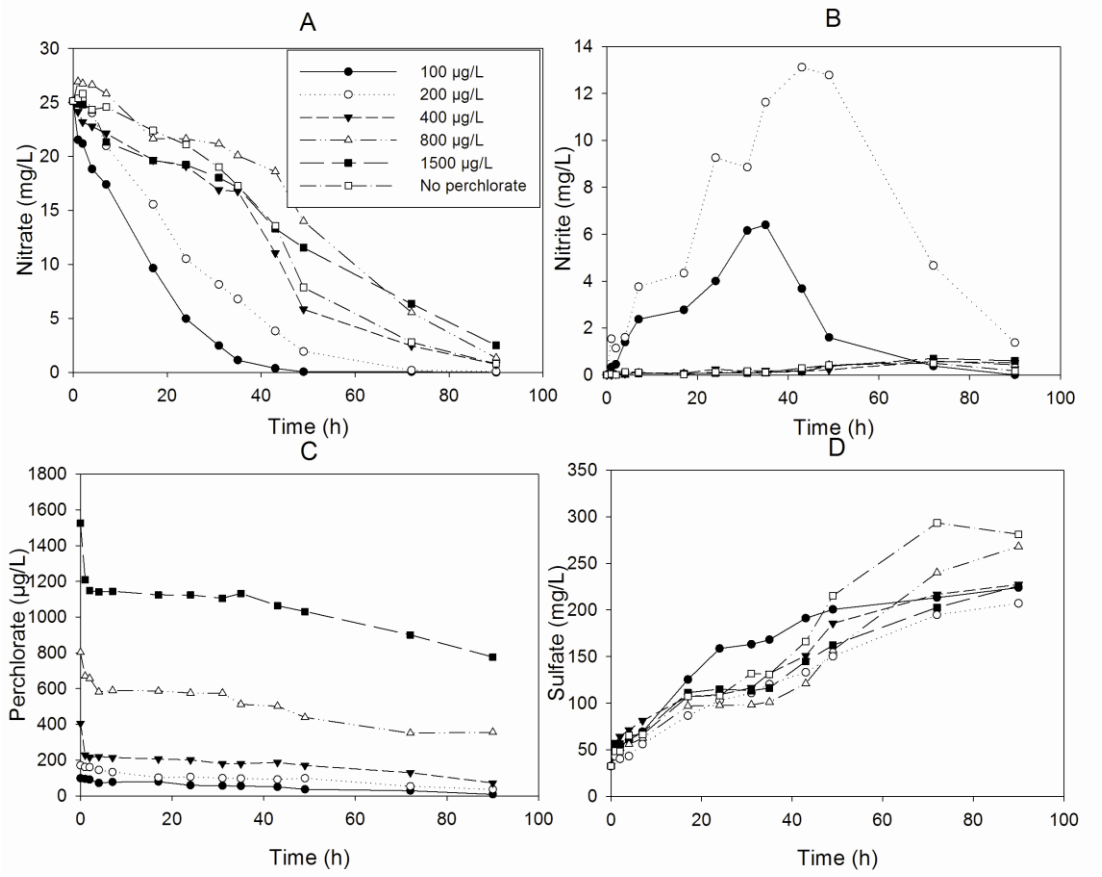

Figure 5.3 : Performances of autotrophic reactors fed with elemental sulfur and limestone (A: Nitrate; B: Nitrite; C: Perchlorate and D: Sulfate concentrations over time).

The perchlorate removal performances of R3-based bioreactors are shown in Figure 5.3. Similar to nitrogen reduction efficiencies, the lowest reduction performance was obtained in this attempt. Influent 98.22 µg/L perchlorate decreased to 8 µg/L by the end of 90h. Increasing influent perchlorate concentration resulted in decreased removal efficiencies. Influent 200 µg/L perchlorate concentration was decreased to 35.54 µg/L corresponding to 17% removal. When influent perchlorate was increased to 1500 µg/L, removal was only 50%.

Although limestone-fed reactors were not as efficient as $NaHCO_3$-fed reactors in our study, many successful results are available where limestone generally is used to provide alkalinity (Ju et al., 2007). Sierra-Alvarez et al., (2007) obtained a maximum denitrification rate of 0.25-0.3 g NO_3^--N/(L.d) in a lab-scale packed-bed bioreactor with sulfur/limestone ratio of 1/1 (Sierra-Alvarez et al., 2007). Limestone is a readily available material and can be easily found in abundance. However its utilization in conventional treatment processes is limited due to its low solubility and increasing effluent hardness. These properties of limestone should be taken into consideration for full-scale applications.

5.4 Conclusions

Three batch assays consisting of six reactors were operated for a 90 hour period to identify the possible toxic effect of perchlorate in the denitrification process. Process kinetics were also evaluated. A methanol-based heterotrophic process reduced both nitrate and perchlorate within 17 and 49 h respectively. Among autotrophic reactors, $NaHCO_3$-fed elemental sulphur-based reactors exhibited a better reduction performance than that of limestone-fed reactors. The buffering acidity with dissolved alkalinity sources resulted in better denitrification and perchlorate reduction performances. Nitrate, nitrite, as well as perchlorate were detected in the effluent of limestone-fed reactors whereas nitrate was completely reduced in 50 hours in $NaHCO_3$-fed reactors. Perchlorate was found to be an inhibiting factor for nitrate reduction, but no clear inhibiting effect was observed.

6. MOLECULAR ANALYSIS

Molecular studies were based on determination of microbial diversity and their quantifications. Firstly DNAs in the samples were extracted and then two different molecular analysis were performed: DGGE - sequencing and real time PCR amplification. In DGGE analysis, samples were amplified with unviersal primers however in real time PCR analysis specific primers for nitrate and perchlorate reducing process were used.

6.1 DNA Extraction

Samples were collected during the all denitrification process in different conditions. They are summarized in Table 6.1 below. DNA was extracted with FastDNA SPIN Kit (MP Bio) for soil according to manufacturer's instructions (Supplementary 1). Extracted DNA samples were stored at -20 ºC. DNA quantification was provided via nanospectrophotometer (IMPLEN, Germany).

DNA concentrations were measured in range of 30 ng/ul -115 ng/ul. A260/280 rates should be 1.8 to say that DNA is pure without protein contaminations. Our results were sufficiently good in this manner. Genomic DNA was also confirmed by 1% (w/v) agarose gel electrophoresis and staining with ethidium bromide. Gel was photographed on Vilber Lourmat Quantum St4 gel documentation system (Figure 6.1).

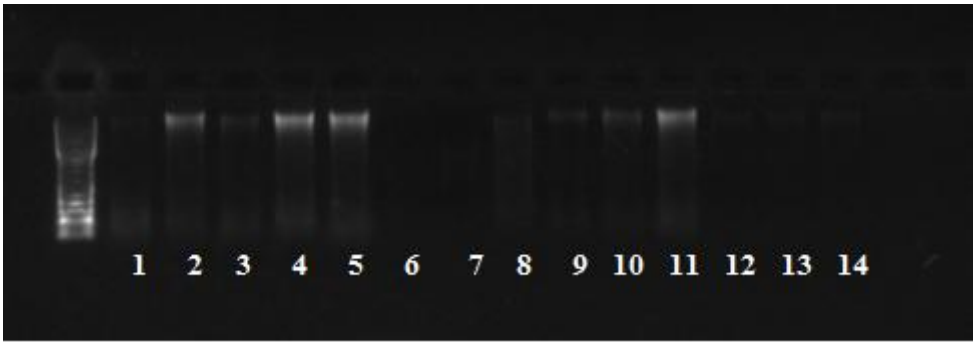

Figure 6.1 : Isolated genomic DNA on agarose gel.

Table 6.1 : Samples and DNA concentrations.

Sampling Number	Reactor condition	DNA conc (ng/ul)	Final DNA conc. (A260/280)
1	$NaHCO_3$ fed Autotrophic R	42.5	2.125
2	Heterotrophic	72.5	2.071
3	Limestone fed Autotrophic R.	50	2.222
4	Autotrophic (Seq. System)	115	1.840
5	Heterotrophic (Seq. System)	102	1.864
6	Mixotrophic (Front)	40	2.000
7	Mixotrophic (Mid)	30	2.000
8	Mixotrophic (Tail)	45	2.000
9	Heterotrophic (Front)	37.5	1.875
10	Heterotrophic (Mid)	40	2.000
11	Heterotrophic (Tail)	77.5	1.824
12	Autotrophic (Front)	30	2.000
13	Autotrophic (Mid)	30	2.000
14	Autotrophic (Tail)	37.5	2.143

6.2 DGGE Analysis

The crude DNA sample was used as a template for PCR. Fragments corresponding to nucleotide positions 341–926 of the *Escherichia coli* 16S rRNA gene sequence were amplified with the forward primer GC-BacV3, to which at the 5' end a GC clamp was added to stabilize the melting behavior of the DNA fragments in the DGGE, and the reverse primer 907r (Muyzer et al., 1996). Primers were shown in Table 6.2. PCR amplification was performed using Thermocycler T3000 (TECHNE) with the following program: initial denaturation at 95 ºC for 5 min followed by 30 cycles of denaturation at 94 ºC for 30 s, primer annealing at 50 ºC for 1 min and primer extension at 72 ºC for 2 min, and final extension at 72 ºC for 10 min. The presence of PCR products was confirmed by 1% (w/v) agarose gel electrophoresis and staining with ethidium bromide prior to DGGE analysis. DGGE was performed with the D-CODE System (BioRAD, The Netherlands). PCR samples of 45 mL were loaded onto 6% (w/v) polyacrylamide gel (acrylamide:bisacrylamide stock solution, Sigma-Aldrich) in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with

denaturing gradient ranging from 40 to 60% (100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60 ºC with 100V for 16 h. After electrophoresis, the gel was stained in an SYBR Gold solution (100 µl/L in TAE) for 30 min and photographed on Vilber Lourmat Quantum St4 gel documentation system.

Table 6.2 : Primers used in DGGE analysis.

Target region	Primers	DNA sequence (5′–3′)
16S rRNA	BacV3f	CCTACGGGAGGCAGCAG
	907r	CCGTCAATTCCTTTRAGTTT
	GC-BacV3f	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCC CCGCCCGCCTACGGGAGGCAGCAG

Bands in DGGE gels were excised with a razor blade and placed in sterile 200 µl vials. DNA was eluted into 20 µL of water and stored over night at 4 ºC. The eluted DNA was used as template in PCR reactions with the primers BacV3f (without GC clamp) and 907r using the same PCR program as described above. The presence of PCR products was confirmed by 1% (w/v) agarose gel electrophoresis and staining with ethidium bromide after DGGE analysis. The sequencing of the purified products was performed at REFGEN (Ankara, Turkey).

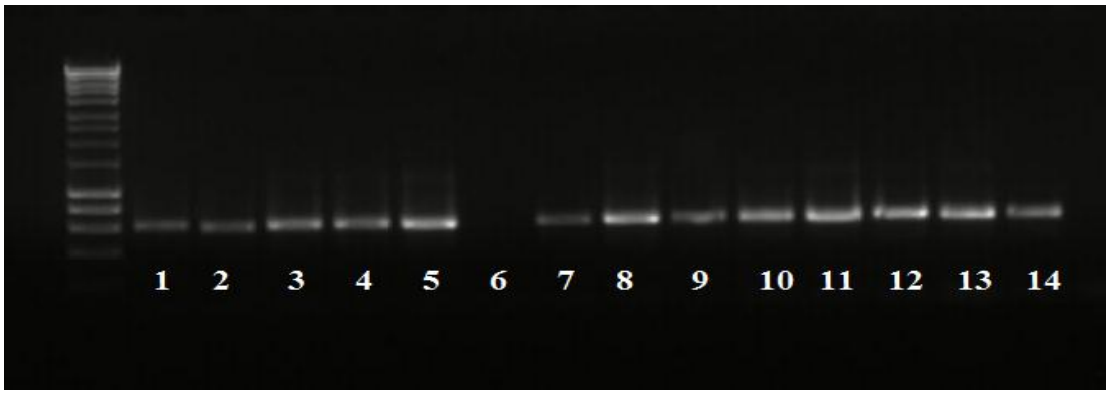

Figure 6.2 : DNA bands after PCR with GC-clamp primers on agarose gel (prior to DGGE).

DGGE analysis was performed in various denaturing gradients to see a wide range of microbial community.

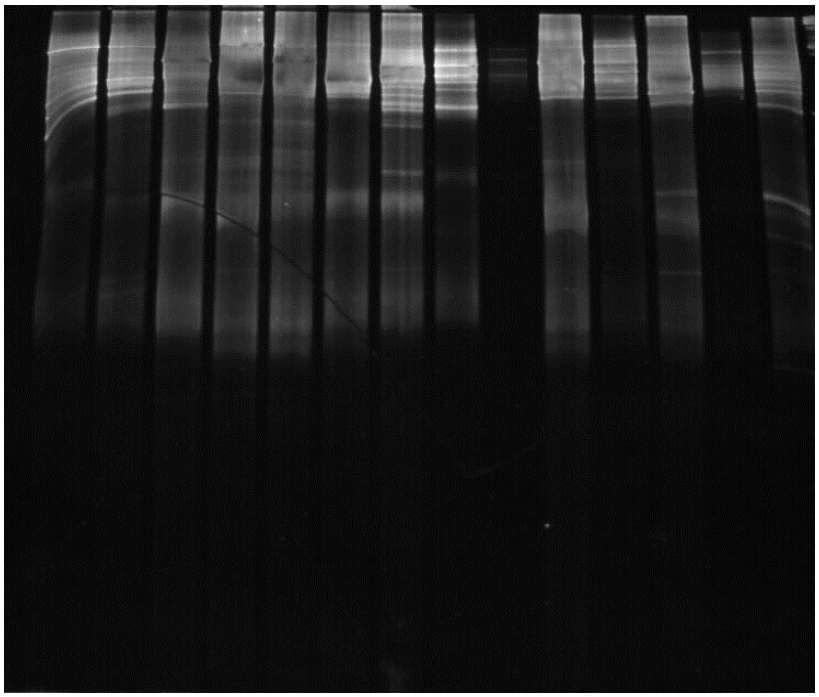

Figure 6.3 : Gradient gel (40-60%).

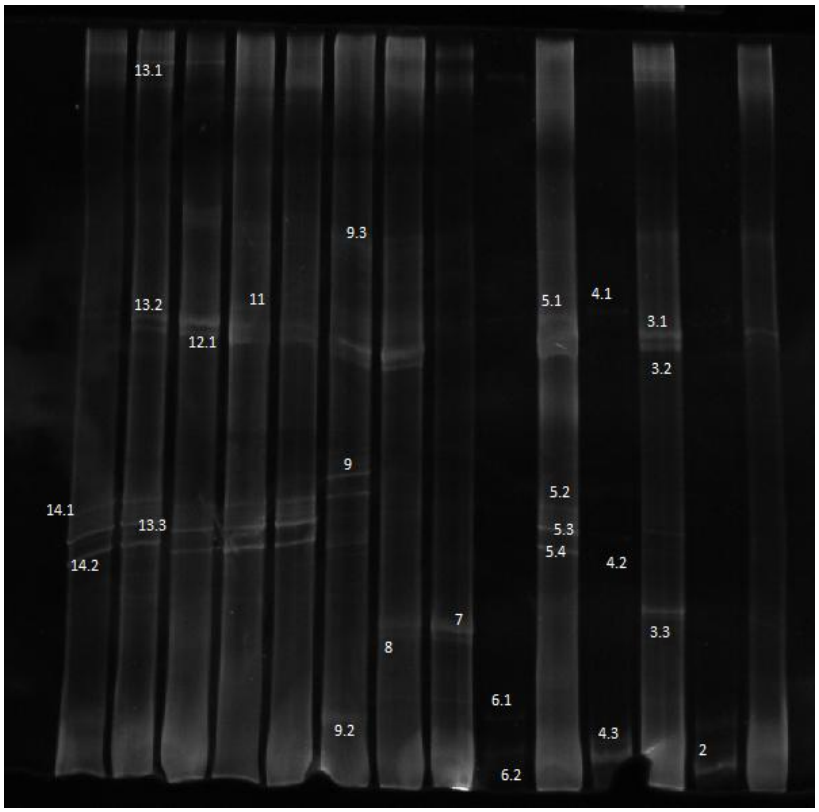

Figure 6.4. Gradient gel (30-50%).

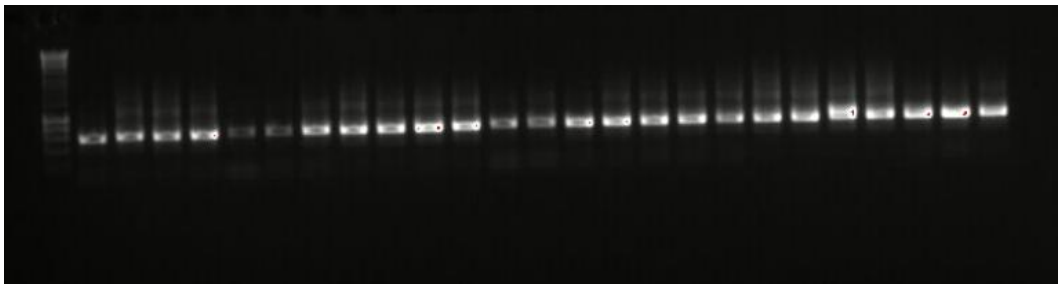

Figure 6.5 : DNA bands after PCR with universal primers on agarose gel (after DGGE).

According to nucleotide blast results, some microbial genus are highlighted. When the all reactor was only fed without perchlorate, genus *Rhodococcus*, *Gordonia, Nocardia* and *Mycobacterium* were found. Results for each reactor are presented in Table 6.3.

Table 6.3 : DGGE results.

Reactor conditions	**Microorganism Genus**
Mixotrophic reactor	*Spirochaeta, Treponema, Ignavibacterium, Rhodospirillum, Liberibacter, Acinetobacter, Psychrobacter, Pseudomonas*
Autotrophic reactor	*Mycobacterium, Bacillus, Rhodococcus,*
Heterotrophic reactor	*Anaerolinea, Dictyoglomus, Acinetobacter, Psychrobacter, Pseudomonas*

6.3 Real Time PCR Analysis

Real time PCR method serves accurate results in the short time period through monitoring DNA amplifications within real time. DGGE analysis has opportunity to give the information on microbial diversity inside the sludge, however determination of microbial species was up to DGGE gradient rates. Therefore, real time PCR supplement the DGGE results since it targets the specific gene region belonged to related species. The method also gives information on quantity of samples. To calculate the exact quantity, standart curves should be formed.

Real time PCR experiments were carried out with FastStart DNA Master SYBR Green I kit (Roche) via the device LightCycler (Roche, Germany), according to manufacturer's instructions and also with some modifications. The following experimental protocol is adapted to our samples after optimization of the cycle parameters as mentioned below.

Table 6.4 : Real Time PCR conditions.

Order	Process	Condition
1	Denaturation temperature	95 °C
2	Pre-incubation time ("activation" of the Fast- Start Taq DNA polymerase	10 min
3	Denaturation	10 s at 95 °C
4	Annealing	10 s at 57 °C
5	Extension	45 s at 72 °C
6	Cycle number	40
7	Final extension	5 min at 72 °C

Temperature transition rate is set as 0°C/s during the experiments. The 20 µl of PCR solution consists of 2 µL 10x Mastermix (Roche); 2 µL 25 mM $MgCl_2$; 1,25 µM from each primers (Table 6.4); pure water (dH_2O) and 2 µL diluted DNA. In all PCR runs, control groups were used to detect unspesific amplifications and all samples were implemented twice to minimize experimental errors. After the amplification with SYBR-Green I dye, melting curves were conducted from ^{o}C 65 to ^{o}C 95^{o}C with elevated gradient of 0,1^{o}C/s. SYBR Green I is a dye, specific for double-stranded DNA. The fluorescence is greatly enhanced by this kind of binding. During each phase of DNA synthesis, the SYBR Green I dye binds to the amplified PCR products and the amplicon is detected by its fluorescence. Combining amplification with melting curve analysis can enhance specificity and sensitivity of amplification reactions. LightCycler Software 4 was used to analyse experiments. Real time PCR analyses belonged to universal primers, perchlorate reductase, chloride dismutase and chlorate reductase (Table 6.5).

Table 6.5 : Primers used in PCR and real-time PCR analysis.

Target region	Primers	DNA sequence (5′–3′)
Perchlorate Reductase	pcrA320F pcrA598R	GCGCCCACCACTACATGTAYGGNCC GGTGGTCGCCGTACCARTCRAA
Dismutase (cld) (154 bp)	Forward Reverse	CACCGCGCTTTGCCTTCAT GAGCCCCGTCGAGTGGTAGAG
Reductase (clrA) (93 bp)	Forward Reverse	GGACGAAGCGCTCACCGAAATC TGCGAAAGGGCGCTTGGGAATA
16s rRNA universal primer	Forward Reverse	CATCGGAACGTGCCCAGTAGTG TGACATCGGCCGCTCCAATAG
narG	Forward Reverse	TCGCCSATYCCGGCSATGTC GAGTTGTACCAGTCRGCSGAYTCSG
napA	V17m napA4r	TGGACVATGGGYTTYAAYC ACYTCRCGHGCVGTRCCRCA

Bacterial contents were determined using the universal primer. It showed that the bacterial content of mixotrophic reactor were lower than that of in other reactors. It was based on the operational time since mixotrophic reactor was only operated for 174 days whereas autotrophic reactors and heterotrophic reactor were operated for 370 days. At the end of this period, $NaHCO_3$ fed autotrophic reactor and heterotrophic reactor were connected for sequential configuration and further operated. Long operational periods should increase the bacterial content in the reactor. Accordingly heterotrophic reactor contained more bacteria than that of in autotrophic reactors as expected.

In other results, all these enzymes are distinctively targeted by the gene regions. In all reactors nitrate reductase enzymes are found in the front side of the reactor whereas all perchlorate reductase enzymes including cld and clA were found in the tail side of the column reactors. Since bacteria prefarably utilizes nitrate first and then perchlorate the enzymes were distributed accordingly. For perchlorate reductase enzyme, % amount for each sampling point is presented in Figure 6.6.

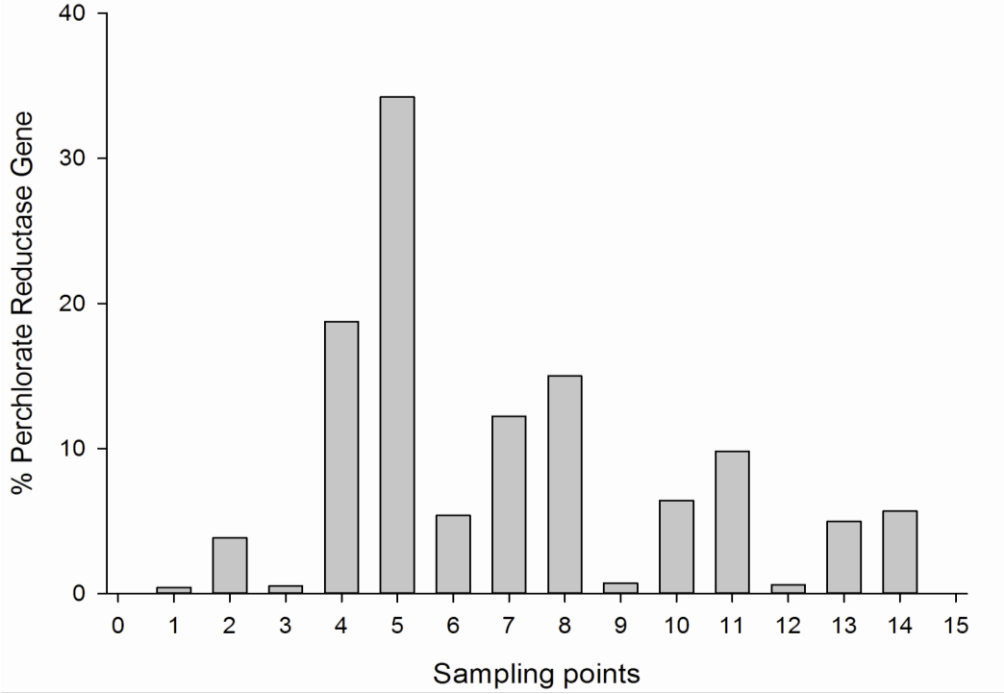

Figure 6.6 : Perchlorate reductase gene rate for the reactors. Sampling points are: (1)$NaHCO_3$ based autotrophic, (2) heterotrophic, (3) limestone based autotrophic, (4) front side of $NaHCO_3$ based autotrophic, (5) front side of heterotrophic, mixotrophic front(6) mid(7) tail(8), heterotrophic front(9) mid(10) tail(11) and autotrophic front(12) mid(13) tail(14). Perchlorate reduction was observed in all parts of the reactors, however enzyme amount was increased in the tail side of the reactors (sampling points 6 - 14).

7. CONCLUSIONS AND RECOMMENDATIONS

The results of the study indicated that complete nitrate reduction is possible with both autotrophic and heterotrophic processes. The mixotrophic reactor also exhibited highly efficient nitrate removal. When simultaneous perchlorate and nitrate reduction was desired, the mixotrophic reactor was the treatment of choice. The mixotrophic reactor allowed for high nitrate reduction rates under high perchlorate loads, while complete perchlorate reduction was achieved. The mixotrophic reactor eliminated the risk of high sulfate production and also decreased the residual organic content by means of DOC. It was observed that the autotrophic reactor fed with a dissolved alkalinity supply ($NaHCO_3$) had better reduction efficiencies than the limestone-supported autotrophic reactor.

The heterotrophic-autotrophic sequential system was also tested under high nitrate load. Nitrate and perchlorate reduction was initiated in the heterotrophic reactor and concluded in the following autotrophic reactor. Autotrophic reactors could be used as a single reactor for the simultaneous reduction of nitrate and perchlorate. They can also works as a secondary treatment unit after a heterotrophic reactor to reduce and oxidize remaining anions and unoxidized substrates in the heterotrophic reactor effluent.

The specific conclusions of the study are summarized below:

1) The maximum perchlorate reduction rate of the autotrophic reactor, which used limestone as alkalinity source was 11.4 mg/(L.d)
2) The maximum perchlorate reduction rate for the $NaHCO_3$ fed autotrophic rector was 11640 mg/(L.d)
3) The autotrophic reactors successfully reduced the 25 mg NO_3^--N/L with a HRT of 2 hours. The nitrate reduction rate was 300 mg/(L.d).
4) The nitrate and perchlorate reduction rates for heterotrophic reactor were 600 and 24 mg/(L.d).

5) The nitrate and perchlorate reduction rates for mixotrophic reactor were 400 and 16 mg/(L.d).

6) The heterotrophic autotrophic-sequential system reduced 100 mg NO_3^--N/L and 1000 µg ClO_4^-/L. Using this system, the maximum reduction rates of nitrate and perchlorate were 1200 and 12 mg/(L.d).

7) According to real time PCR analysis, perchlorate reducers were located in all sides of the reactors, however abundant in the tail side of the reactors.

The recommendations upon the results of this thesis could be summarized as:

1. The biological treatment of nitrate and perchlorate could not only used directly to groundwater, but also could use for the brine produced by membrane processes.
2. For in situ treatment applications, low temperature kinetics (at 10-15 °C) should be determined and applied to monitor reduction performances.
3. The gas production in reactors may be collected and analyzed with a gas chromatography to identify its content. The NO_X (NO and NO_2) or N_2O content should be considered carefully as these gases are air pollutants and greenhouse gases.

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