Denitrification performance and microbial community dynamics in a denitrification reactor as revealed by high-throughput sequencing

Sevgi Demirel

ABSTRACT

The dynamics of the bacterial community associated with the denitrification process in a fixed bed column reactor (FBCR) were investigated using 454-pyrosequencing methodology. A FBCR filled with elemental sulfur and limestone was operated for about 94 days under autotrophic and mixotrophic (autotrophic + heterotrophic) conditions at 30 °C. Efficient simultaneous bromate and nitrate removal was achieved at feed concentrations of 500 μg/L bromate and 45 mg/L nitrate under autotrophic and mixotrophic conditions. Operational taxonomic units-based analysis (97% similarity cut-off) of bioreactor samples (three periods) revealed that the microbial diversity changed regardless of operational conditions. *Sulfurimonas* spp. was dominant in the reactor at the adaptation stage. *Thiobacillus denitrificans* is a chemolithoautotrophic bacterium that is capable of the oxidation of inorganic sulfur compounds. After the adaptation period, the microbial profile changed such that *Spirochaetacea* spp. and *Denitratisoma* spp. were major species in the column reactor. After 60 d of operation, *Hyphomicrobium vulgare* became dominant due to the mixotrophic denitrification conditions.

Key words | bromate, high-throughput sequencing, microbial community, mixotrophic denitrification, nitrate

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INTRODUCTION

Bromate (BrO_3^-) can be formed during ozonation processes of natural water containing bromide (Br^-) (Gunten & Pinkernell 2000). Bromate is a drinking water pollutant that represents a risk for public health because of its carcinogenic potential. Therefore, the maximum allowable bromate concentration in drinking water has been set as 10 μg/L by the US Environmental Protection Agency (USEPA) and European Community Drinking Water Directive (Clark & Bouting 2001). In drinking water resources, nitrate and bromate can be present together, as nitrate is a common co-contaminant in surface and ground waters (Westerhoff & James 2001). The USEPA limits the maximum contaminant level of 10 mg/L nitrate for drinking water (Ucar *et al.* 2016). Nitrate can be found in drinking water resources also contaminated with bromate; however, bromate is usually present at low concentrations (<100 μg/L). It is a challenge to develop a remediation technology able to economically remove these pollutants from contaminated drinking water resources. Biological denitrification is a cost-effective process for removing nitrogenous compounds from water. Biological denitrification, either autotrophic or heterotrophic, has been proposed separately for nitrate and/or bromate removal. Our previous study (Demirel *et al.* 2013) showed that sulfur-based autotrophic and mixotrophic denitrification can be considered as an alternative process for nitrate and bromate removal from drinking

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water. In the sulfur-based autotrophic denitrification process, sulfur and nitrate act as an electron donor and an acceptor, respectively, without requirement of organic supplementation (Reaction (1)). Comparing to the electron donors, elemental sulfur (S^0) as an attractive alternative electron donor possesses the characteristics of being non-toxic, water insoluble, stable under normal conditions, and readily available (Sahinkaya *et al.* 2013).

$$55\,S^0 + 50NO_3 + 20\,CO_2 + 38\,H_2O + 4\,NH_4^+ \rightarrow 4\,C_5H_7NO_2 + 55\,SO_4^{2-} + 25\,N_2 + 64\,H^+ \quad (1)$$

The main disadvantages of the autotrophic denitrification process are sulfate and acid generation. Sahinkaya *et al.* (2011) showed that elimination of sulfate generation by sulfur-based autotrophic denitrification processes is possible by stimulating a simultaneous autotrophic and heterotrophic (mixotrophic) denitrification process by methanol supplementation. This process minimizes individually the disadvantages of heterotrophic and autotrophic denitrification: the former generates alkalinity which the latter consumes; the result is an alkali-neutral reaction (Demirel *et al.* 2013).

In this research, an autotrophic and mixotrophic fixed bed column reactor (FBCR) was investigated to remove bromate and nitrate simultaneously. The objectives of this research were to (1) determine which autotrophic and heterotrophic denitrifying bacteria in the FBCR could reduce bromate to below the emission standard of 10 μg/L treatment objectives and (2) compare the microbial community structure under different operational conditions by using pyrosequencing technology.

Table 1 | Operation conditions used in FBCR

Stages	Period	Days	Bromate feed (μg/L)	NO_3-N feed (mg/L)	HRT (h)	Methanol feed (mg/L)[a]
Start-up	1	0–10	–	45	10.1	–
Autotropic	2	10–60	500	45	10.1	–
Mixotrophic	3	60–94	500	45	10.1	75 (29.5)

[a]Value in parentheses represents the methanol concentration as dissolved organic carbon (DOC).

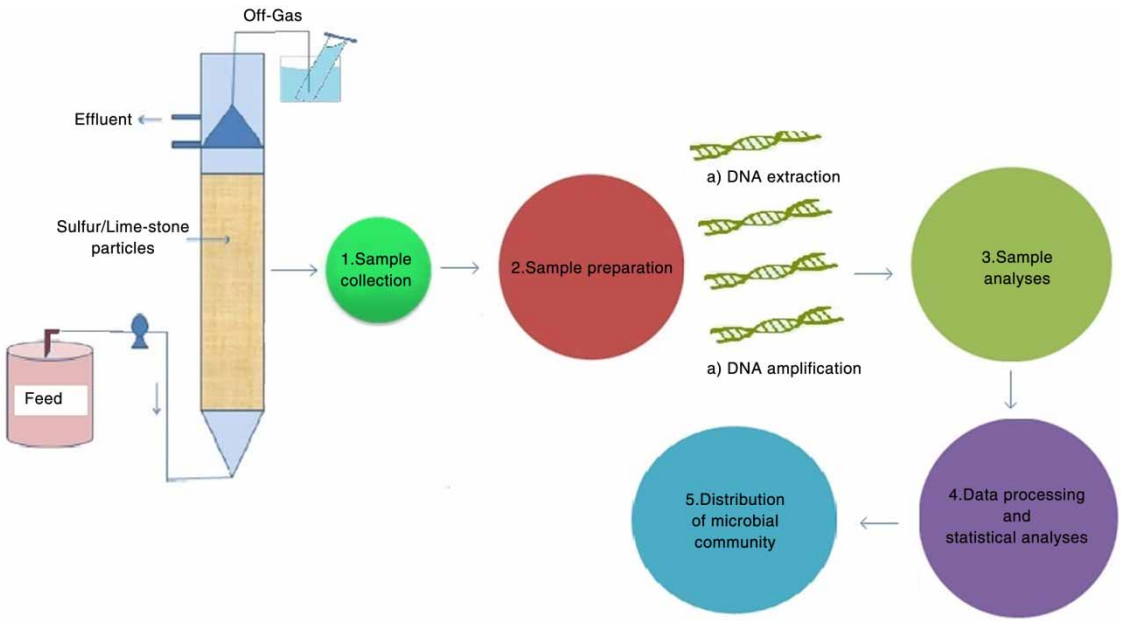

Figure 1 | Scheme showing the application of 454-pyrosequencing analysis to the study of bacterial communities associated with denitrification.

METHODS

Design of column reactor and analytical methods

For a complete removal of nitrogenous compounds (NO_3-N and NO_2-N), denitrification conditions were provided in a laboratory-scale glass column reactor with an empty volume of 500 ml filled with elemental sulfur (0.5–1 mm) and limestone (0.5–1 mm) in ratios of 2/3 and 1/3, respectively. The inoculum was obtained from the first anoxic tank of a five-stage Bardenpho process. The reactor was supplemented with methanol during mixotrophic denitrification conditions. Hydraulic retention time (HRT) was calculated considering the empty bed volume (Table 1).

Samples for analysis were collected from the reactor effluent at least three times a week for the measurement of NO_3^-, NO_2^-, bromate, bromide and pH. The feed solution was sampled once a week for the determination of NO_3^-, NO_2^-, bromate, bromide and pH. All samples were filtered over a 0.45 μm-pore-size sterile filter and stored at 4 °C until analysis. NO_3^-, NO_2^- bromate and bromide concentrations were determined by ion chromatography. For identifying the changes in the microbial communities in the reactor, three different biomass samples (during the adaptation, autotrophic and mixotrophic periods) were taken from the sampling port located in the lower part of the reactor (Figure 1).

PCR amplification and high-throughput 16S rRNA gene pyrosequencing

DNA was extracted using a PowerSoil DNA isolation kit (MoBio) according to the manufacturer's instructions. Extracted DNA samples were stored at −20 °C until downstream applications. After linear amplification of DNA using the REPLI-G Mini Kit (Qiagen), amplified DNA samples were used for amplification of the bacterial V4 region of the 16S rRNA gene by using forward primer bac515f (GTGCCAGCMGCCGCGGTAA) and reverse primer bac806R (GGACTACVSGGGTATCTAAT). The polymerase chain reaction (PCR) mix contained 250 ng template DNA, 25 mM dNTP, 10× buffer with $MgSO_4$, 2.5 U *Pfu* polymerase, and 0.2 μM of each primer in a 100 μL reaction mixture. The temperature conditions for PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 3 min followed by 28 cycles of denaturation at 94 °C for 30 sec, primer annealing at 53 °C for 40 sec and primer extension at 72 °C for 1 min. The reaction was terminated after 5 min extension at 72 °C. Pyrosequencing of amplicons was performed on a Roche 454 Genome Sequencer FLX Titanium according to the manufacturer's protocol. The obtained DNA sequence was compared to search the closest 16S rRNA sequences of the reference microorganisms available in GenBank by BLAST.

RESULTS AND DISCUSSION

Nitrate and bromate removal performance in long-term operation

The column reactor was first operated under start-up conditions for 10 days (period 1, Table 1) at 45 mgNO_3^--N/L.

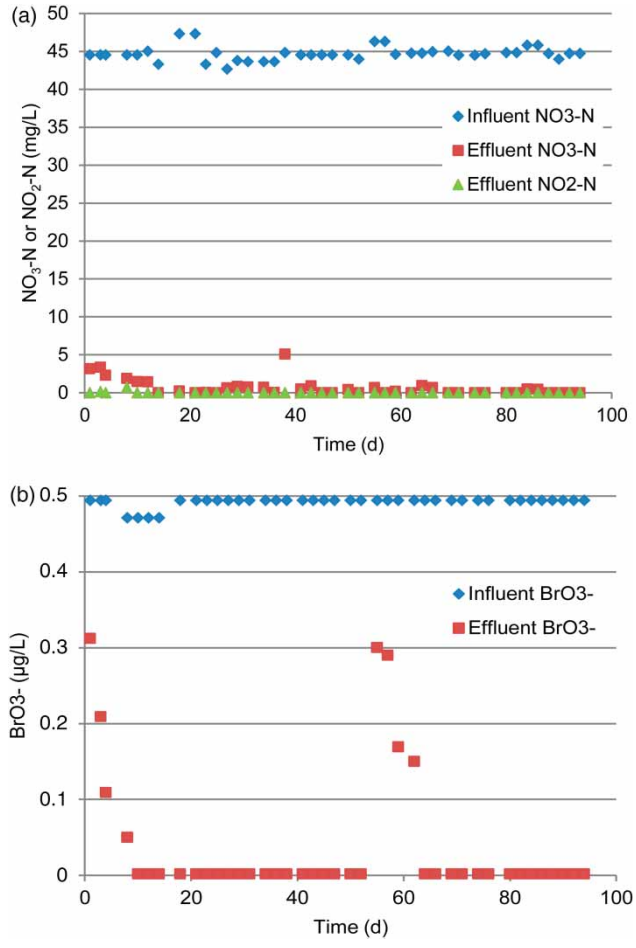

Figure 2 | Influent and effluent (a) NO_3-N or NO_2-N, and (b) BrO_3^- variations.

After the adaptation period, the column reactor was operated under autotrophic conditions up to 60 days at 45 mg NO_3^--N/L and 500 μg BrO_3^-/L. The reduction of bromate and nitrate was monitored during these periods (Figure 2). In the first 10 days of the operation of the autotrophic period, both nitrate and bromate were detectable in the effluent, although each decreased linearly to undetectable levels. Under steady-state conditions, the bromate concentration was less than 3 μg/L. The measured bromide concentration in the effluent demonstrated that all the bromate was stoichiometrically reduced without any by-product formation under steady-state conditions. Moreover, bromate and nitrate were removed simultaneously from the water, without detectable nitrite in the effluent.

After day 60, the FBCR was fed with methanol (75 mg/L) in addition to 45 mg NO_3^--N/L and 500 μg/L bromate (Table 1). Similarly to the autotrophic operating conditions, bromate and nitrate were removed under the mixotrophic conditions. During the operation period, a hydraulic residence time of 10.1 h was sufficient for complete nitrate and bromate transformation to dinitrogen gas and bromide, respectively (Figure 2).

Composition of the microbial community

In recent years, scientists have been understanding the structure and function of target communities and clarifying the link between them by new-generation microbial ecology techniques. New-generation molecular approaches have enabled deeper and more accurate analysis of complex ecosystems, leading to significant progress in identifying active species in the environment (Baytshtok *et al.* 2009).

Pyrosequencing analysis yielded a total of 43,105 sequence reads from three genomic DNA samples after removal of short and low-quality reads. The 454-pyrosequencing results indicated that microbial community structure changed through the periods depending on operational conditions (electron donor, energy source because of changing conditions

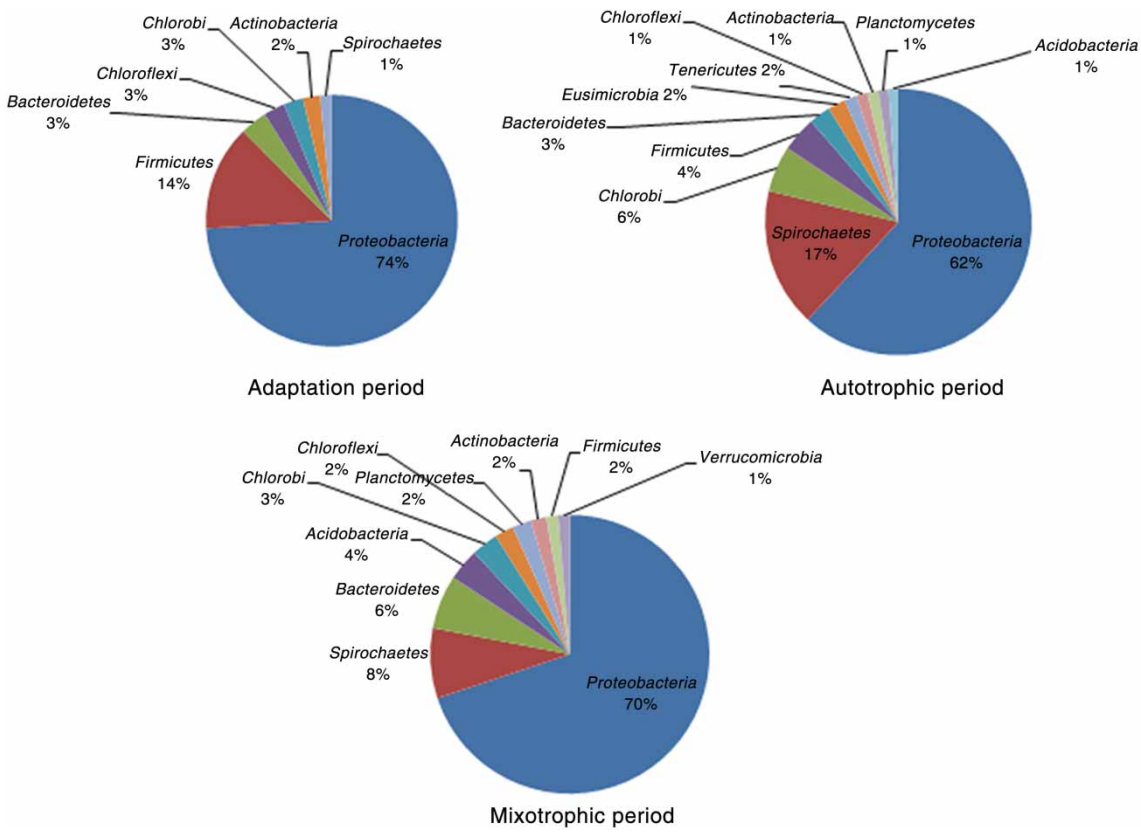

Figure 3 | Relative abundance of the main phyla identified in the sulfur-based FBCR. Only phyla with a relative abundance greater than 1% are shown.

such as autotrophic or mixotrophic etc.) in the FBCR. *Proteobacteria* was the main phylum in all operational stages standing out in the presence of denitrifiers (Figure 3). This bacterial composition was consistent with the previous study which investigated the microbial diversity of different operational conditions on the effect of denitrification (Park *et al.* 2011). The *Proteobacteria* accounted for 71.05%, 58.15% and 65.95% of the sequences which were related to the known bacteria during each of the operational stages, respectively. In addition *Bacteroidetes* and *Chloroflexi* were phyla common to all periods. Despite the limited knowledge about the role of *Chloroflexi* in denitrification, it might be related to reducing nitrate to nitrite (Kohno *et al.* 2002). *β*-Proteobacteria represented the most abundant class in all operational periods (21.52%, 24.81% and 31.51% for adaptation, autotrophic and mixotrophic stages, respectively). A functional role for members of *β*-proteobacteria in denitrifying biofilm has been suggested by Wrighton *et al.* (2010).

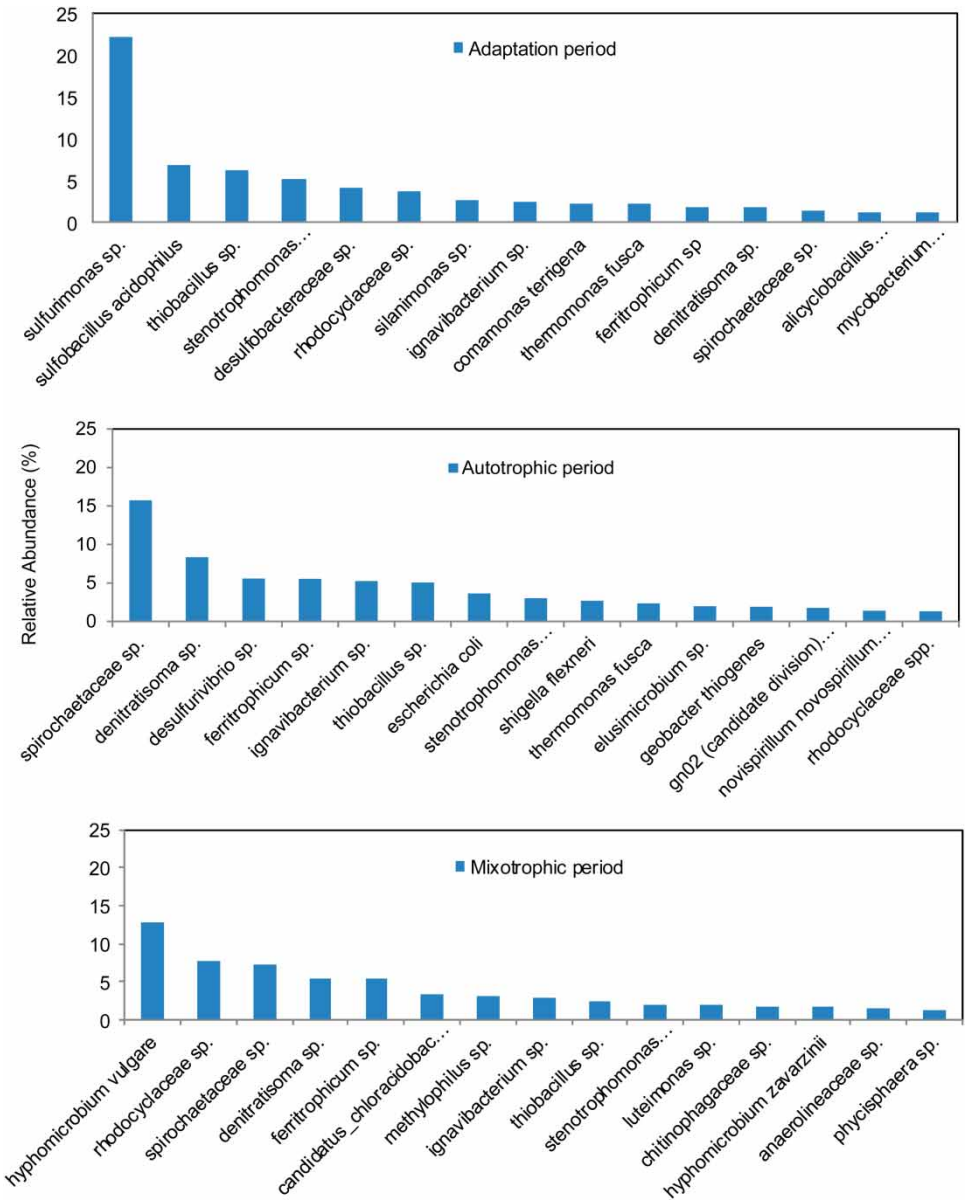

Figure 4 | Relative abundance of the main species identified in the sulfur-based FBCR. Only species with a relative abundance greater than 1% are shown.

As seen in Figure 4, *Sulfurimonas* sp. was the most abundant species (22.11%) in the reactor at the adaptation stage. *Sulfurimonas* was the most commonly reported autotrophic denitrifier, which reduced nitrate to N_2 while oxidizing elemental sulfur or reduced sulfur compounds to sulfate (Koenig *et al.* 2005; Shao *et al.* 2010). These bacteria in the column reactor should be responsible for sulfur-based autotrophic denitrification at the adaptation stage. After the adaptation period, the column reactor was operated at 500 μg/L of bromate concentration under autotrophic conditions. At this stage, the microbial profile changed so that *Spirochaetaceae* sp. (15.72%) and *Denitratisoma* sp. (8.33%) were major species in the column reactor. *Denitratisoma* have been playing an important role in the denitrification of nitrate as in the references reported by Mao *et al.* (2008, 2010). The distribution and intensity of the samples taken at different stages seemed to be identical, which can be explained by ion distribution data, which was consistent with patterns of nitrate and nitrite reduction in the column reactor. A similar distribution of predominant bacterial species in denitrification columns has also been reported (Koenig *et al.* 2005).

After day 60 (period 3), the FBCR was fed with methanol (Table 1). The facultative methylotrophs, e.g., *Hyphomicrobium*, and *Methylophaga*, which prominently belong to *β*-proteobacteria, were dominant species when methanol was added during the mixotrophic stage. These populations can be classified as obligate (growing on C1 compounds as sole source of carbon and energy only) and facultative methylotrophs (growing on C1 and multi-carbon compounds) (Lu *et al.* 2014). In this stage, *Hyphomicrobium vulgare* was the most abundant species (12.68%) due to the heterotrophic denitrification conditions (Figure 4). *H. vulgare* has been shown to grow anaerobically using nitrate as an alternative terminal electron acceptor and methanol as a sole carbon and energy source (Nikolausz *et al.* 2005).

The microbial activity in the bioreactor depends mainly on three steps: (i) sulfur particles (electron donor) must be microbially oxidized, (ii) nitrate (electron acceptor) must be microbially reduced, and (iii) bromate (via the co-metabolic action of nitrate reductase) must be reduced in a specific reduction pathway. In the mixotrophic denitrification ecosystem, the sulfate-reducing bacteria utilized the sulfate produced by the autotrophic denitrifiers, and the alkalinity generated in the heterotrophic denitrification could effectively compensate for alkalinity consumption by the autotrophic denitrification. Although the composition of the microbial community might reflect the source of the bacteria, it is believed that the experimental conditions must have led to the development of a stable and specialized community of denitrifying bioreactor.

CONCLUSION

A laboratory-scale mixotrophic denitrification column, using elemental sulfur (in the autotrophic stage) and sulfur/methanol (in the mixotrophic stage) as an electron donor, was successfully operated for over 94 days. During the operation, the performance and microbial community structure of a FBCR with combined autotrophic and mixotrophic denitrification was investigated. The 454-pyrosequencing analysis showed that the dominant species were *Sulfurimonas* spp. in the reactor at the adaptation stage. When bromate was added to the reactor, the microbial profile changed so that *Spirochaetacea* spp. and *Denitratisoma* spp. were major species in the column reactor. The 454-pyrosequencing analysis revealed that the autotrophic and heterotrophic bacteria coexisted in the mixotrophic environment.

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