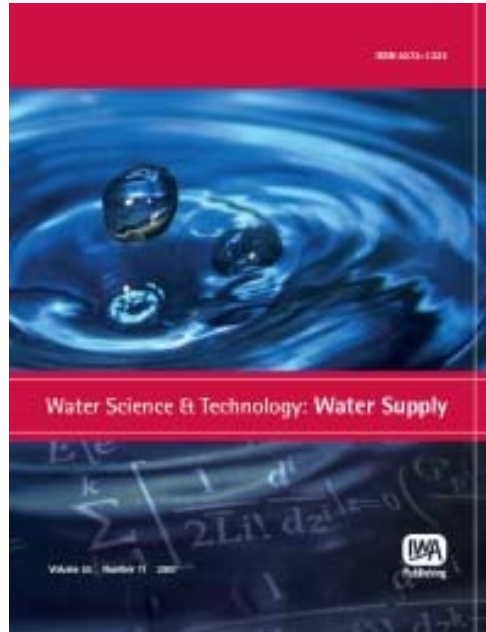


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Evidence of specialized bromate-reducing bacteria in a hollow fiber membrane biofilm reactor

K. J. Martin, L. S. Downing and R. Nerenberg

ABSTRACT

Bromate is a carcinogenic disinfection by-product formed from bromide during ozonation or advanced oxidation of drinking water. We previously observed bromate reduction in a hydrogen-based, denitrifying hollow fiber membrane biofilm reactor (MBfR). In this research, we investigated the potential existence of specialized bromate-reducing bacteria. Using denaturing gradient gel electrophoresis (DGGE), we compared the microbial ecology of two denitrifying MBfRs, one amended with nitrate as the electron acceptor and the other with nitrate plus bromate. The DGGE results showed that bromate exerted a selective pressure for a putative, specialized bromate-reducing bacterium, which developed a strong presence only in the reactor with bromate. To gain further insight into the capabilities of specialized, bromate-reducing bacteria, we explored bromate reduction in a control MBfR without any primary electron acceptors. A grown biofilm in the control MBfR reduced bromate without previous exposure, but the rate of reduction decreased over time, especially after perturbations resulting in biomass loss. The decrease in bromate reduction may have been the result of the toxic effects of bromate. We also used batch tests of the perchlorate-reducing pure culture, *Dechloromonas* sp. PC1 to test bromate reduction and growth. Bromate was reduced without measurable growth. Based on these results, we speculate bromate's selective pressure for the putative, specialized BRB observed in the DGGE was not growth related, but possibly based on resistance to bromate toxicity.

Key words | biological reduction, bromate, hollow fiber membrane

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INTRODUCTION

Bromate is a disinfection by-product formed from bromide during the ozonation or advanced oxidation of drinking water. The US Environmental Protection Agency (EPA) and the European Union regulate bromate, a Group 2 carcinogen, at 10 µg/L in drinking water (Butler *et al.* 2005a). Bromate is not considered to be naturally occurring, though in a study of 36 river samples, bromate concentrations ranged from 4–8 µg/L (Kruithof & Meijers 1995). In treated water, bromate concentrations have been found as high as 150 µg/L (Krasner 1993). Bromate may also enter drinking water through other pathways. A recent study found up to 3 µg/L bromate in finished waters, attributed to bromate contained in the hypochlorite disinfectant (Weinberg *et al.*

2003). Also, bromate was recently detected in open water reservoirs in Los Angeles; the unexpected bromate was believed to have formed from bromide, chlorine, sunlight, and dissolved oxygen (Kemsley 2007).

Water treatment plants using ozone have few cost-effective options to remove the highly soluble, stable bromate ion. Abiotic processes, such as nanofiltration, UV irradiation, high energy electron beams, and reducing agents, can be impractical due to high capital costs, high energy requirements, concentrated waste streams, and lower finished water quality (Butler *et al.* 2005a). Biological reduction may be one of the most cost-effective treatment technologies. Significant bromate reduction has been

achieved using biologically activated carbon (Kirisits & Snoeyink 1999; Kirisits *et al.* 2001, 2002), a suspended growth bioreactor (Butler *et al.* 2005b), a fixed-film bioreactor (Butler *et al.* 2006), and a hollow fiber membrane biofilm reactor (Downing & Nerenberg 2007).

Despite the effectiveness of biological reduction for treating bromate, little is known about the microbial bromate reduction process. Bromate should be a highly favorable electron acceptor with a standard redox potential exceeding that of oxygen (Milazzo & Caroli 1978), yet little or no growth is observed on bromate, possibly due to formation of the toxic intermediate bromite (BrO_2^-) (van Ginkel *et al.* 2005b; Downing & Nerenberg 2007). Oxygen, nitrate, and nitrite inhibit bromate reduction (Hijnen *et al.* 1999; Kirisits & Snoeyink 1999; Downing & Nerenberg 2007), and self-inhibition is observed at high bromate concentrations (van Ginkel *et al.* 2005b; Downing & Nerenberg 2007). Literature suggests that bromate reduction occurs as a cometabolic reaction with the nitrate or chlorate reductase. Mixed cultures and pure cultures of denitrifying bacteria reduce bromate (Hijnen *et al.* 1995; Kirisits & Snoeyink 1999; Downing & Nerenberg 2007). Similarly, van Ginkel *et al.* (2005a) showed cometabolic reduction of bromate by chlorate-respiring microorganisms.

Literature has provided limited evidence of specialized, bromate-reducing bacteria (BRB), which are defined in the work presented here as bacteria capable of bromate reduction. In a pure culture study with bromate provided as the sole electron acceptor, the denitrifying bacterium *Pseudomonas fluorescens* strain P17 (ATCC 49642) reduced bromate without previous enrichment (Hijnen *et al.* 1995). However, Downing & Nerenberg (2007) did not observe bromate reduction in a pure culture biofilm of *Ralstonia eutropha* (ATCC 17697), a denitrifier that expresses both periplasmic and membrane bound nitrate reductase enzymes. These results support the existence of specialized BRB among denitrifying bacteria. Further evidence of specialized BRB include bromate removal rates that increased with time in a suspended growth bioreactor (Butler *et al.* 2005b), and enriched activated sludge that reduced bromate, but not nitrate, chlorate, or perchlorate (van Ginkel *et al.* 2005b). However, it is not clear if this culture actually grew on bromate.

In this research, we investigated the potential existence of specialized bromate-reducing bacteria via studies on

continuous, mixed-culture reactors and via batch tests on a pure culture.

METHODS

MBfR

Each MBfR, similar to those used in previous studies (Nerenberg & Rittmann 2004), included 16 hollow-fiber membranes (HFM200TL, Mitsubishi Rayon, Japan) housed in a glass tube (Figure 1). The hollow fibers provided 30.5 cm^2 of surface area. The tube was part of a recirculation loop, which provided completely mixed conditions. We pressurized the hollow fibers at 35 kPa with hydrogen gas. The liquid holdup was 25 cm^3 .

Denitrifying MBfR

Two identical reactors were run simultaneously; one with nitrate as the electron acceptor (“nitrate reactor”) and another with nitrate plus bromate (“bromate reactor”). The reactors ran on a synthetic medium comprised of 1.386 g Na_2HPO_4 , 0.849 g KH_2PO_4 , 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.025 g $(\text{NH}_4)_2\text{SO}_4$ per litre, as well as a trace mineral solution (Nerenberg *et al.* 2002). The medium was fed at a rate of 0.5 mL/minute, resulting in a hydraulic retention time (HRT) of 50 minutes. A recirculation rate of 150 mL/minute provided well mixed conditions in the reactor. Both reactors were inoculated with biomass from a previous bromate-reducing reactor. Therefore, the inoculum had been preselected for possible BRB. After 21 days of running both reactors with nitrate only, we introduced

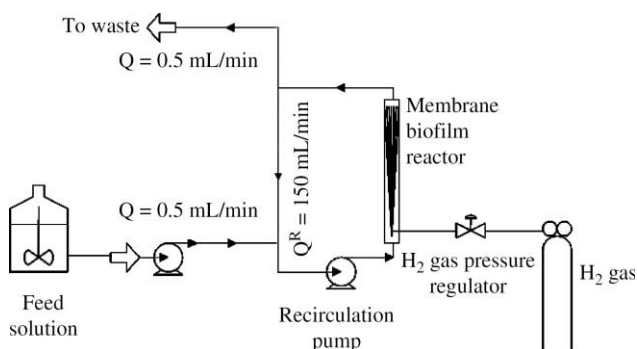


Figure 1 | Experimental set-up of the MBfR.

bromate concentrations of 0.1 mg/L for 22 days to the bromate reactor. Bromate concentrations were subsequently increased to 1.5 mg/L and 5.0 mg/L as shown in Table 1. At day 0, 21, 50, and 95, we sampled the biomass from each reactor.

Control MBfR

An additional control MBfR was operated with no influent nitrate or bromate. The membranes were pressurized with 60 kPa hydrogen. The reactors ran on a synthetic medium comprised of 0.346 g Na₂HPO₄, 0.212 g KH₂PO₄, 0.170 MgCl₂·6H₂O, 0.040 g (NH₄)Cl, 0.088 g Na₂CO₃, 1 mL trace mineral solution, and 1 mL Ca-Fe solution per litre (Nerenberg *et al.* 2002). The synthetic medium was fed at a rate of 1 mL/minute, resulting in an HRT of 25 minutes. The reactor was well mixed, with a recirculation rate of 150 mL/minutes. We inoculated the reactor with biomass from a previous bromate-reducing, hydrogen-based MBfR. After 125 days of operation with no electron acceptors, the influent was amended with 1 mg/L bromate. The medium was sparged with N₂ to remove dissolved oxygen, and stored in carboys with a pressurized N₂ headspace.

Batch tests

Batch tests of a pure culture perchlorate-reducer, *Dechloromonas* sp. PC1 (Nerenberg *et al.* 2006), were conducted in 160-mL glass serum bottles sealed with thick, butyl rubber stoppers and aluminium crimps. Synthetic medium as described for the control MBfR and bromate or chlorate stock solutions were filter sterilized with sterile 0.2 µm nylon filters and added to the capped, autoclaved bottles. In one test, PC1 was grown on 130 mg/L chlorate; in the second test, 2.5 mg/L bromate served as the available

electron acceptor. To ensure anaerobic conditions, the headspace was successively vacuum degassed and pressurized with 95% H₂/5% CO₂ three times. The bottles were incubated at approximately 20°C on a shaker table at 120 rpm. The inoculum consisted of PC1 grown on chlorate, washed twice, and suspended in synthetic medium. We inoculated and sampled the bottles in an anaerobic glove chamber (COY, Grass Lake, MI) using sterile needles and syringes. The optical density (OD) was tested using spectrophotometer at 600 nm (ThermoSpectronic, Rochester, NY).

Analytical methods

Bromate, bromide, nitrate, nitrite, chlorate, and chloride concentrations were measured via ion chromatography with a sodium hydroxide eluant (IC2500, AS19/AG19 and AS11/AG11 columns, method detection limit of 5 µg/L for bromate, Dionex Corporation, Sunnyvale, CA).

Molecular methods

DNA from the MBfR was extracted using the PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, CA). DNA fragments from the 16S rRNA gene were then amplified by PCR using the general bacterial primers 341F-GC and 907R (Muyzer *et al.* 1993). Denaturing gradient gel electrophoresis (DGGE) was conducted to compare the microbial ecology among the samples (BioRad, Hercules, CA) (Muyzer *et al.* 1993).

Clone libraries were created to compare the ecology of the nitrate and bromate MBfRs. A nested PCR was used to obtain template for cloning. First, we carried out PCR on the 16S rRNA gene using primers 27F and 1492R (Gurtler & Stanisich 1996). Secondly, we performed PCR using the 341F-GC-clamp and 907R primers to obtain the template for cloning, which was completed with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). We used the Geneious Pro Software (Drummond *et al.* 2007) to manipulate our sequences and the Ribosomal Database Project II Classifier (Wang *et al.* 2007) to obtain preliminary classification results.

Table 1 | Nitrate and bromate concentrations schedule for the denitrifying reactors

Day	Nitrate reactor		Bromate reactor	
	Nitrate (mgN/L)	Bromate (mg/L)	Nitrate (mgN/L)	Bromate (mg/L)
Start-up (21 days)	5	0	5	0
1–21	5	0	5	0.1
22–50	5	0	5	1.5
51–95	5	0	5	5.0

RESULTS AND DISCUSSION

Performance and microbial ecology of the denitrifying MBfRs

In both denitrifying reactors, nitrate was quickly reduced to below $20\ \mu\text{gN/L}$. At steady state, the bromate reactor reduced 100 and $1,500\ \mu\text{g/L}$ bromate to below the $10\ \mu\text{g/L}$ maximum contaminant level (Figure 2), with stoichiometric increases in bromide. When the influent bromate concentration was increased to $5\ \text{mg/L}$, the effluent bromate increased from 0.1 to $3\ \text{mg/L}$ over approximately 35 days (Figure 3), suggesting that the higher influent bromate was inhibitory. This is consistent with findings by Downing & Nerenberg (2007).

DGGE provides a semi-quantitative “fingerprint” of the microbial community in a sample. Ideally, each band in the DGGE represents a species of bacteria, and the relative intensity of the band represents that species’ relative prominence within a community. Figure 4 shows DGGE results for biofilm collected from the nitrate and bromate reactors at 0, 21, 50, and 95 days.

At Day 0, after the 21 days of startup time, the nitrate reactor (N) and bromate reactor (B) had been running under identical conditions. Therefore, they should have had similar DGGE banding patterns. Small differences in banding patterns existed for N0 and B0, most notably the missing band highlighted by the lower dashed box. DGGE results could have differed based on variations in the

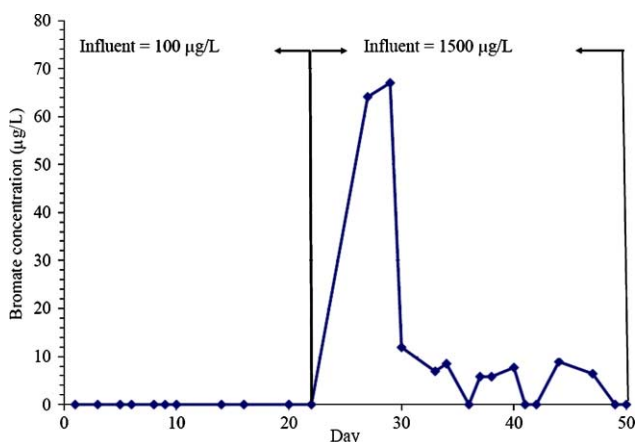


Figure 2 | Bromate reactor effluent concentrations from day 0–50.

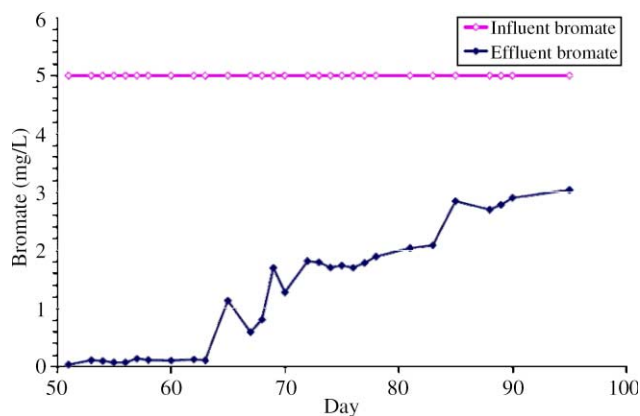


Figure 3 | Bromate reactor influent and effluent concentrations from day 50–day 95.

biomass collected for DNA extraction, which would have been exaggerated through the PCR.

At Day 21, 50, and 95, the nitrate concentration remained at $5\ \text{mgN/L}$ in both reactors while the bromate concentration was increased stepwise in the bromate reactor. The top band highlighted in Figure 4, strong in both N0 and B0, decreased in the nitrate reactor and remained dominant in the bromate reactor. This could be explained by the presence of a specialized BRB from the bromate-reducing inoculum that was lost in the denitrifying reactor and maintained in the bromate reactor. If there was a selective pressure for BRB under bromate-reducing conditions, it could be based on its growth on bromate, or on its higher resistance to potential bromate toxicity.

Preliminary cloning results

Clone libraries were created for biofilm samples collected from both reactors on Day 95, i.e., corresponding to lanes N95 and B95 in Figure 4). Using the Ribosomal Database Project II Classifier, we obtained preliminary classification of 44 clones from Sample N95 and 43 clones from Sample B95. As shown in Table 2, three phyla were common to both samples: Acidobacteria, Bacteroidetes, and Proteobacteria. Most clones were classified under the phylum Proteobacteria, with the vast majority of these falling under the class Betaproteobacteria, the order Burkholderiales, and the family Comamonadaceae. Further classification finds approximately 75% of the Proteobacteria in both samples are of the genus *Hydrogenophaga*, which is appropriate as hydrogen served as the sole electron donor.

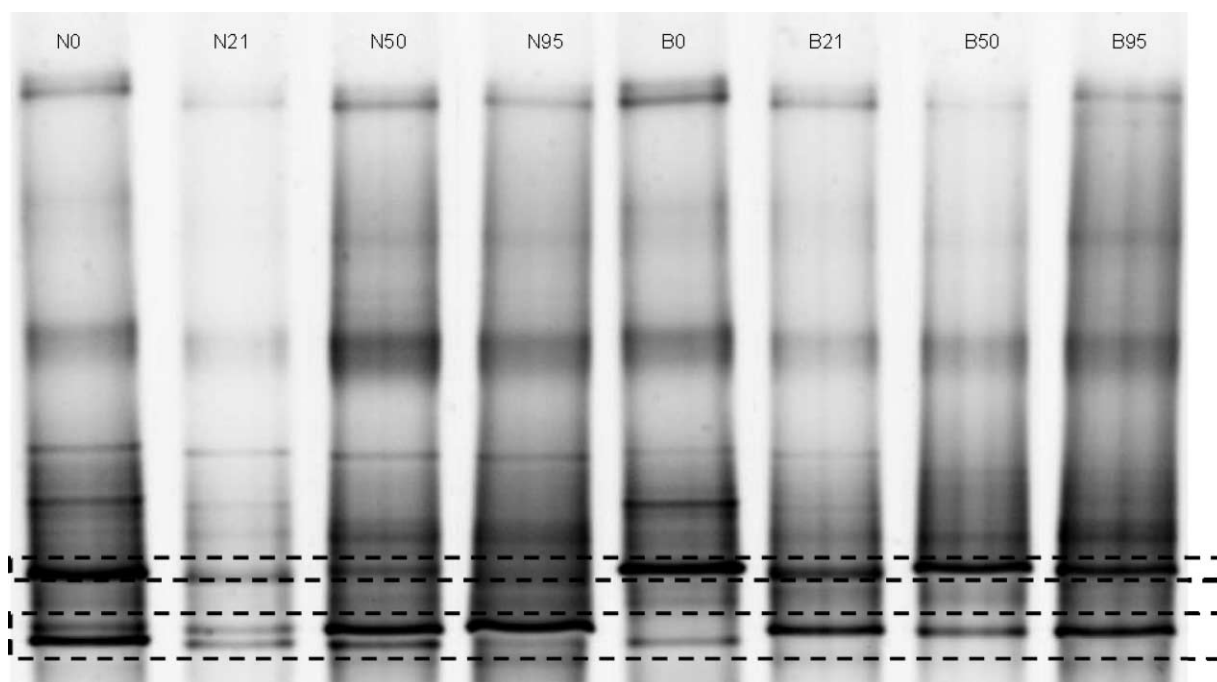


Figure 4 | DGGE results for the nitrate and bromate reactors. Each lane is either denoted by an N for “Nitrate reactor” or a B for “Bromate reactor,” followed by a number indicating the day.

Interestingly, the N95 clone library was strongly dominated by Proteobacteria, while the B95 clone library was mainly comprised of Proteobacteria and Acidobacteria. Perhaps the DGGE band that remains strong only in the bromate reactor (top dashed box) consists of Acidobacteria, and the strong band in both reactors (bottom dashed box) consists of Hydrogenophaga.

Control reactor results

Despite having no nitrate, oxygen, or bromate added to the influent of the control MBfR, some biofilm growth was observed. This is most likely due to trace amounts of oxygen, although growth on trace amounts of nitrate, sulfate, or bicarbonate is also possible. On Day 125, 1 mg/L of bromate

was added to the control reactor, and within 24 hours, 69% of the bromate was reduced. Bromate reduction occurred without previous exposure to bromate, although the reactor’s initial inoculum was from a bromate-reducing bio-reactor. Bromate reduction gradually decreased over time, especially after perturbations that resulted in small losses of biomass. Decreasing rates of bromate reduction over time may have been the result of the toxic effects of bromate, slowing the growth of the cometabolic biofilm. The BRB in this reactor may have been facultative denitrifiers that used oxygen as a terminal electron acceptor under anoxic conditions, as suggested for a MBfR for perchlorate reduction (Nerenberg *et al.* 2008).

Batch test results

The reduction of chlorate and bromate was tested in serum bottles with the perchlorate-reducer *Dechloromonas* PC1. Within four days, PC1 reduced the initial 130 mg/L chlorate to chloride, and the OD at 600 nm increased from 0.008 to 0.055 percent absorbance (A) (Figure 5). The OD slowly decreased once chlorate was completely degraded. Bromate was reduced from 2.5 mg/L to 1.5 mg/L over nine days, but

Table 2 | Distribution of clone libraries among the classification level of phylum

Phylum	N95 sample (Percentage of clone library)	B95 sample (Percentage of clone library)
Acidobacteria	4.5	35
Bacteroidetes	4.5	7
Proteobacteria	91	56
Unclassified	0	2

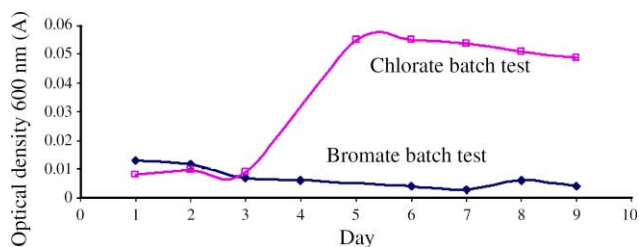


Figure 5 | Changes in OD in PC1 batch tests with chlorate and bromate. While the OD of PC1 increased, indicating growth, the OD in the batch test with bromate decreased over time.

the OD never increased. During this bromate reduction, the OD decreased from 0.13 to 0.04 A. Lack of full reduction of 2.5 mg/L bromate over nine days and continuous loss in biomass suggests PC1 is incapable of measurable growth on bromate.

CONCLUSION

Bromate reduction occurred in denitrifying MBfRs, but also in MBfRs without any bromate or nitrate. Relatively low bromate concentrations inhibited bromate reduction under denitrifying conditions. Perchlorate reduction occurred in a pure culture of a perchlorate-reducing bacterium with bromate as the sole electron acceptor, but no growth was observed. Differences in microbial ecology between the denitrifying reactor and a denitrifying reactor with bromate suggest that bromate exerts a selective pressure, but probably not based on growth with bromate. Selection may be based on resistance to bromate toxicity. Ongoing work will further analyze the clone library of the DGGE samples and test pure cultures of denitrifiers to study the ability of denitrifying bacteria to reduce bromate. With a better understanding of BRB, biological treatment options for the reduction of bromate can be developed further.

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