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Cite this: *Environ. Sci.: Water Res. Technol.*, 2016, 2, 1049

Evaluation of electron donors for biological perchlorate removal highlights the importance of diverse perchlorate-reducing populations†

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This research investigated the treatment of a synthetic groundwater with approximately 100 mg L⁻¹ perchlorate (ClO₄⁻) and 15 mg L⁻¹ nitrate (NO₃⁻-N) using a bench-scale, fluidized-bed bioreactor (FBR). The groundwater was amended sequentially with acetate and MicroC2000™, a proprietary, glycerol-based electron donor. Nitrate reduction to less than 0.05 mg L⁻¹ NO₃⁻-N and perchlorate removal to less than 0.3 mg L⁻¹ ClO₄⁻ occurred under both electron donor regimes, although a higher biomass yield was observed and a higher influent COD concentration was required to maintain the same effluent quality when MicroC2000™ was used as the electron donor. High-throughput sequencing of partial 16S rRNA genes from biomass collected at several time points revealed that a single *Dechloromonas* population dominated the perchlorate-reducing community under both electron donors and *Dechloromonas* species comprised greater than 30% relative abundance of the bacterial community by the end of reactor operation. The same *Dechloromonas* population was abundant in two bench-scale systems fed lower perchlorate concentrations, although several other perchlorate-reducing bacteria, presumably with higher affinities for perchlorate, were also abundant in those systems. The results suggest that to reduce perchlorate to levels that allow groundwater to serve as a drinking water source, distinct environments for diverse perchlorate-reducing bacteria with high and low affinities for perchlorate are needed. Such conditions can be created by using two bioreactors in series.

Received 20th July 2016,
Accepted 2nd October 2016

DOI: 10.1039/c6ew00181e

rsc.li/es-water

Water impact

Biological removal of perchlorate and nitrate from a highly contaminated groundwater can be accomplished by feeding acetate or a proprietary electron donor to a bioreactor system. To reduce perchlorate to levels that allow groundwater to serve as a drinking water source, distinct environments for perchlorate-reducing bacteria with high and low affinities for perchlorate are needed in the bioreactor system.

1. Introduction

Perchlorate (ClO₄⁻) has been used as an oxidant in various industrial and defense-related applications, including the manufacturing of solid missile and rocket fuel, air bags, and road flares.^{1–3} Uncontained disposal of perchlorate-contaminated wastewater, made possible by the lack of a federal drinking water standard or cleanup requirement, has resulted in widespread environmental contamination across the United States.⁴ A 2007 study reported that 395 sites across

35 states, the District of Columbia, and two commonwealths had measurable levels of perchlorate in drinking water, groundwater, surface water, and sediment or soil.⁴ In 11 of these sites, perchlorate concentrations exceeded 500 mg L⁻¹ and the highest level reported (3.7 g L⁻¹) was in groundwater. The highest perchlorate concentrations were found in Arkansas, California, Nevada, Texas and Utah. Outside of the United States, reported perchlorate concentrations of contaminated groundwater and surface waters generally have been less than 1 mg L⁻¹.^{5–8}

Human exposure to perchlorate is a health concern for reproductive-age women because perchlorate decreases thyroid hormone production and adversely affects fetal development.⁹ Exposure to perchlorate occurs primarily through ingestion of contaminated tap water and food.¹⁰ Ingestion of contaminated tap water was estimated to increase total perchlorate intake levels in reproductive-age women in the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ew00181e

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United States by 3–24%.¹¹ These estimates were based on measurements of perchlorate concentrations in drinking water from the United States Environmental Protection Agency (U.S. EPA) UCMR1 database and data from other regional studies of perchlorate contamination. Additionally, the use of perchlorate-contaminated groundwater for irrigation increases the risk of human ingestion of perchlorate because several crops can absorb and accumulate perchlorate from irrigation waters.¹² Certain food items, such as dairy products, fruits and vegetables, have been found to significantly contribute to perchlorate exposure.¹³ For these reasons, remediation of perchlorate-contaminated waters is important to limit human exposure to perchlorate and protect public health.

Biological reduction is an established approach for remediation of perchlorate-contaminated waters.¹⁴ Dissimilatory perchlorate-reducing bacteria are facultative anaerobes that can use perchlorate or chlorate as terminal electron acceptors for anaerobic respiration.¹⁵ Through this metabolism, perchlorate is reduced by the perchlorate reductase enzyme to chlorate (ClO_3^-), which is subsequently reduced by perchlorate reductase to chlorite (ClO_2^-).¹ Chlorite dismutase detoxifies chlorite by converting it to chloride (Cl^-) and molecular oxygen. The innocuous nature of the products of biological perchlorate reduction and the low chemical input needed to support biological treatment¹⁶ make biological remediation of groundwater containing high concentrations of perchlorate more attractive than technologies that employ chemical reducing agents.^{3,17,18}

Biological water treatment often takes place in biofilm systems, such as fixed-bed bioreactor and fluidized-bed bioreactor (FBR) systems.¹⁹ In an FBR, biofilm develops on a support medium (e.g., granular activated carbon [GAC]) that is suspended or fluidized within the bioreactor. Biological perchlorate removal requires the addition of an electron donor to provide sufficient reducing equivalents to reduce perchlorate. An electron donor is also needed to reduce oxygen, the energetically more favorable electron acceptor, and the commonly co-occurring contaminant nitrate (NO_3^-).^{20,21} Suitable electron donors in such applications include acetate, ethanol, lactate and hydrogen.^{1,22} A variety of alternative electron donors are also available, including proprietary electron donors such as MicroC products (Environmental Operating Solutions, Bourne, MA). MicroC products have been used as electron donors for denitrification in wastewater treatment.^{23–26} In particular, several municipal and industrial denitrifying wastewater treatment plants are using MicroC2000™, a glycerol-based electron donor with an undisclosed composition. However, MicroC2000™ has not been studied for perchlorate removal from groundwater. Feasibility studies of diverse electron donors are important for providing the water treatment industry with flexibility for electron donor choice.

We recently reported using two bench-scale bioreactors (one fixed-bed bioreactor and one FBR) for treatment of a synthetic groundwater containing $200 \mu\text{g L}^{-1} \text{ClO}_4^-$ and $15 \text{mg L}^{-1} \text{NO}_3^-$ as N.²⁷ Perchlorate reduction was established in

both bioreactors with acetate as the electron donor and then acetate was replaced with MicroC4000™, a commercial, carbohydrate-based product. Biomass yields increased during operation with MicroC4000™ and more reducing equivalents (or chemical oxygen demand (COD)) were required than acetate COD to achieve comparable effluent quality. In the present study, we tested the feasibility of replacing acetate with a glycerol-based MicroC product (MicroC2000™) for treatment of a synthetic perchlorate-contaminated groundwater in a bench-scale FBR. In this case, the influent perchlorate levels were much higher (100mg L^{-1}) than in our previous study.²⁷ First, contaminant removals and estimates of biomass yield under the two electron donor regimes were determined. Then the bacterial community structure in this “high-perchlorate” FBR was characterized by pyrosequencing of 16S ribosomal RNA (rRNA) genes from samples collected during operation with acetate and MicroC2000™. Additionally, biomass was collected from the “low-perchlorate” bench-scale systems reported in our previous study²⁷ to compare perchlorate-reducing populations across three bioreactors that were inoculated in the same way but were operated with different perchlorate loadings and using different MicroC products. While nitrate and perchlorate removals in the low-perchlorate systems were described previously,²⁷ their bacterial community structures are reported here for the first time.

2. Materials and methods

2.1 Reactor setup and operation

An FBR consisting of a glass column, 4.9 cm inner diameter and 56 cm height, fitted with a 3 L bulb at the top, was operated to treat a simulated groundwater containing $100 \text{mg L}^{-1} \text{ClO}_4^-$ and $15 \text{mg L}^{-1} \text{NO}_3^-$ -N. The column was packed with GAC particles (bituminous F816, 0.9 mm effective diameter, 1.25g cm^{-3} true density, and 0.69g cm^{-3} bulk density) collected from a pilot-scale nitrate- and perchlorate-removing bioreactor that used acetate as the electron donor²⁰ (stored at $4 \text{ }^\circ\text{C}$ under aerobic conditions for several years) to achieve a settled bed height of 34 cm. The settled and fluidized bed volumes were 640 and 1075cm^3 , resulting in empty bed contact times (EBCT) of 42.7 and 80.5 minutes, respectively. Excluding a few brief interruptions due to mechanical problems, the FBR was operated continuously for 222 days at $20.5 \pm 0.4 \text{ }^\circ\text{C}$ in a temperature-controlled room. A second, identical FBR and a fixed-bed bioreactor system, which were described previously,²⁷ were operated in parallel to treat a simulated groundwater containing approximately $200 \mu\text{g L}^{-1} \text{ClO}_4^-$ and $15 \text{mg L}^{-1} \text{NO}_3^-$ -N.

Synthetic groundwater simulating a groundwater in Rialto (CA) (except for a higher perchlorate concentration) was prepared (Table 1). The groundwater recipe was amended with ammonium and phosphate to provide sufficient nitrogen and phosphorus for microbial growth and a trace metal solution, SL-10 (Table S1†), which included molybdenum, a trace element required for perchlorate reduction.²⁸ The groundwater was prepared using deionized water and chemical stocks that



Table 1 Chemical composition of the influent synthetic groundwater designed by Li and colleagues (2010)²⁰ according to the composition determined for a real groundwater in Rialto, CA

Chemicals used	Target concentration (mg L ⁻¹)	Expressed as
NaNO ₃	15	NO ₃ ⁻ -N
NaCl, MgCl ₂ ·6H ₂ O and CaCl ₂	36.3	Cl ⁻
K ₂ CO ₃	6	CO ₃ ²⁻
NaHCO ₃	213.5	HCO ₃ ⁻
Na ₂ SO ₄	12.5	SO ₄ ²⁻
NH ₄ Cl	12	N
NaClO ₄	100	ClO ₄ ⁻
KH ₂ PO ₄	2.60	P
Trace element solution SL-10	See Table S4	

were sterilized by filtration or autoclaving. The influent tank was covered to limit deposition of microbes from the air. The synthetic groundwater was pumped into the bottom of the FBR at a flow rate of 15 mL min⁻¹ using a peristaltic pump. The majority of the water that passed through the FBR was recirculated from the top to the bottom at a flow rate of 1.8 L min⁻¹ using a peristaltic pump to allow expansion of the GAC from approximately 34 cm to 44 cm (*i.e.*, 29% expansion before biomass growth). The bed height fluctuated over time and attained a final expansion of 88% (expanded bed height was 64 cm at the end of the study). During reactor operation with acetate, the liquid in the bulb of the FBR was stirred periodically with a brush to resuspend the solids deposited on the sides and to remove excess biomass deposited in the bulb. After the electron donor was changed from acetate to a glycerol product, the reactor maintenance protocol was changed to include daily mixing and wasting of the liquid in the bulb.

2.2 Reactor inoculation

The GAC was inoculated with 70 mL of a suspended culture of *Azospira suillum* JPLRND (formerly called *Dechlorosoma suillum* JPLRND), a strain capable of using oxygen, nitrate, and perchlorate as terminal electron acceptors.²⁹ The strain was isolated from a groundwater sample collected from an aquifer located near the Jet Propulsion Laboratory in Pasadena, California, USA.²⁹ Additionally, the FBR was seeded with approximately 7 g of biologically active carbon (BAC) particles collected from a bench-scale fixed-bed bioreactor operated for nitrate and arsenic removal.³⁰ The bench-scale nitrate and arsenic removing system had originally been seeded with BAC from a system operated for nitrate and perchlorate removal.²⁰ To establish the microbial communities in the filter beds, synthetic groundwater containing nitrate, sulfate, perchlorate (approximately 200 µg L⁻¹), salts, and trace metals was recirculated through the reactors for two days.

2.3 Electron donor

The electron donor was pumped into the influent line using a peristaltic pump to achieve target influent chemical oxygen

demand (COD) levels. Acetate was supplied as the electron donor during the first 114 days of operation. Assuming a net biomass yield of 0.4 g COD_{biomass} g⁻¹ COD_{acetate} (ref. 31) for all electron acceptors and ammonium-N as the nitrogen source, 190 mg L⁻¹ acetate COD is required to remove 15 mg L⁻¹ NO₃⁻-N, 100 mg L⁻¹ ClO₄⁻, and 39 mg L⁻¹ DO (including 7 mg L⁻¹ DO in the influent and 32 mg L⁻¹ DO resulting from the complete reduction of 100 mg L⁻¹ ClO₄⁻). On day 114, acetate was replaced with MicroC2000™, a glycerol product with a COD of 1086 g L⁻¹ in the originally delivered aqueous product. This glycerol product was dosed at a target COD level equivalent to the acetate COD fed during days 81–114, although the measured influent COD concentration was somewhat higher (216.2 mg L⁻¹ C on day 142). Over time, electron donor limitation in the reactor was suspected and the influent concentration of the glycerol product was increased to 491.1 mg L⁻¹ C (average of two data points) on day 142. To minimize the sulfate reduction observed after increasing the influent COD, the influent COD was lowered on day 197 to approximately 442 mg L⁻¹ C.

2.4 Chemical analyses

The reactors were operated for 222 days and the performance was monitored for 217 days. Influent and effluent pH, effluent oxidation reduction potential (ORP), and influent DO concentrations were monitored regularly. DO levels were measured in the influent tank using a WTW multi340 meter with Cellox325 sensors (Weilheim, Germany) (detection limit 0.01 mg L⁻¹). pH was measured using a Seven Easy pH meter (Mettler Toledo, Columbus, OH) and ORP was measured in flow-through cells using an Oakton pH meter (Oakton Instruments, Vernon Hills, IL) fitted with an ORP electrode.

Samples were collected from the influent when a new influent batch was prepared (every 3–4 days) and samples were collected from the effluent approximately every other day. Influent samples were taken from the influent tank and effluent samples were collected from the top of the reactor through a tube positioned at the liquid control point from the top of the bulb (Fig. 1). The samples were filtered through 0.22 µm filters (Fisher, Pittsburgh, PA) and stored at 4 °C until analysis.

Two ion chromatography systems were used to measure concentrations of anions in the FBR influent and effluent. Influent acetate, nitrate, chloride, and sulfate concentrations were measured using a Dionex DX 100 ion chromatograph (Dionex, Sunnyvale, CA) with an AS-14 column (4 × 250 mm) equipped with a conductivity detector, as previously described,³⁰ and carbonate/bicarbonate eluent. Influent and effluent perchlorate concentrations and effluent concentrations of acetate, nitrate, chloride and sulfate were determined using a second ion chromatograph, a Dionex ICS-2100, with a conductivity detector. The anions were chromatographically separated using a Dionex AS-16 column (2 × 250 mm) and a gradient flow of potassium hydroxide (KOH) was used to elute the anions through the column. The second ion



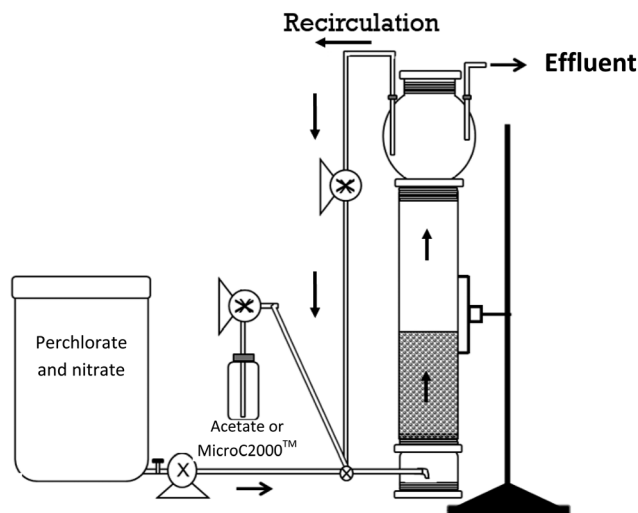


Fig. 1 Setup of the bench-scale, fluidized-bed reactor.

chromatography system had a longer run time and better sensitivity for perchlorate analysis. The method detection limits for acetate, chloride, nitrate as N, sulfate and perchlorate were determined to be $100 \mu\text{g L}^{-1}$, $40 \mu\text{g L}^{-1}$, $50 \mu\text{g L}^{-1}$, $40 \mu\text{g L}^{-1}$ and $3 \mu\text{g L}^{-1}$, respectively.

Soluble non-purgeable organic carbon (sNPOC) was measured in effluent samples using a Total Organic Carbon Analyzer TOC-V CSH (Shimadzu, Columbia, MD) (method detection limit $0.3 \text{ mg L}^{-1} \text{ C}$). All samples were sparged and acidified in the instrument to remove inorganic carbon.

Affluent samples were occasionally analyzed for soluble chemical oxygen demand (sCOD). The HACH COD micro digestion method (HACH, Loveland, CO) for low range COD ($0\text{--}150 \text{ mg L}^{-1} \text{ C}$) was used for sample digestion and COD concentrations were measured colorimetrically using a HACH DR/4000 spectrophotometer at 420 nm (method detection limit $3.7 \text{ mg L}^{-1} \text{ C}$). For time periods when few effluent sCOD measurements were taken, sCOD values were estimated using sNPOC measurements and the relationship between sNPOC and sCOD for effluent samples (Fig. S2†).

2.5 Biomass yield calculations

Biomass yields ($\text{g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{electron donor}}$) were estimated for four different electron donor operating conditions distinguished by composition and/or influent COD concentration and corresponding to data collected during days 102–114, 130–142, 178–189 and 200–210. The time periods chosen to estimate yields for conditions 1 and 2 corresponded with the final 12 days of operation for each condition. Earlier periods were chosen for conditions 3 and 4 because of limited effluent sCOD or sNPOC measurements (Fig. S2†). Data from the first days after a change in condition were not included in any yield estimates to avoid transition periods. Yield was estimated based on COD consumed (*i.e.*, influent COD – effluent COD) and the calculated COD requirement for the amount of perchlorate, nitrate and DO removal observed (sul-

fate reduction was considered if it occurred). The COD consumed beyond the amount required to reduce perchlorate, nitrate, and DO (and sulfate for days 178–189) was considered to be used for biomass growth. Yield was estimated as the ratio of COD used for biomass to total COD consumed. COD equivalents of $2.86 \text{ mg COD mg}^{-1} \text{ NO}_3^- \text{ N}$, $0.67 \text{ mg COD mg}^{-1} \text{ ClO}_4^-$, and $0.322 \text{ mg COD mg}^{-1} \text{ SO}_4^{2-}$ were used in the calculations. Estimates of yield for other time periods for comparison are presented in Table S2.†

2.6 Biomass sampling, DNA extraction and 16S rRNA gene sequence analysis

Biomass samples were collected from the FBR and a low-perchlorate FBR²⁷ on days 86, 96, 189, 196, and 222 by removing BAC particles from the top of each column. Biomass samples were collected from a low-perchlorate fixed-bed bio-reactor²⁷ on days 86 and 189. A composite sample was prepared for each sampling day by removing BAC particles from each of the sampling ports along the depth of the fixed-bed reactor and mixing the BAC particles. Samples were flash-frozen and stored at $-80 \text{ }^\circ\text{C}$. Total DNA was extracted from biomass samples using a phenol–chloroform–isoamyl (25:24:1 at pH 8) extraction protocol.³² The V3–V5 region of bacterial 16S rRNA genes was amplified using primers 563F-modified and 909R.³³ PCR products were pooled based on equal DNA concentrations and submitted for titanium 454-pyrosequencing at the University of Illinois Keck Center.

Sequences were analyzed using mothur version 1.36.1.³⁴ The mothur implementation of AmpliconNoise³⁵ was used to reduce sequencing errors and PCR single base errors. Each sequence was trimmed after the first 450 flows and sequences with any mismatches to the barcode or more than one mismatch to the primer were removed. Sequences with homopolymers longer than 8 bp and sequences less than 200 bp long were also removed. Alignment to the SILVA SSU database version 123 was performed using the Needleman–Wunsch algorithm (*ksize* = 8) and chimeras were removed using UCHIME.³⁶ This resulted in 65 524 sequences that were approximately 200 bp long for further analysis.

Taxonomic assignment of individual sequences was based on the naïve Bayesian method and the reference Greengenes taxonomy database with a confidence score threshold of 80%. Sequences were grouped into operational taxonomic units (OTUs) using the average neighbor approach at three percent sequence divergence. The consensus taxonomy for each OTU was determined using the mothur classify.otu command and the Greengenes taxonomy database.

3. Results and discussion

3.1 Perchlorate and nitrate removal

A bench-scale FBR was fed synthetic groundwater containing $100 \text{ mg L}^{-1} \text{ ClO}_4^-$ and $15 \text{ mg L}^{-1} \text{ NO}_3^- \text{ N}$ and operated under two electron donor regimes. After demonstrating consistent contaminant removal with acetate as the electron donor, acetate was replaced with MicroC2000™, a proprietary, glycerol-



based product. Fig. 2 presents the influent and effluent perchlorate, nitrate and sulfate concentrations after start-up and optimization of operating conditions (days 81–217). As expected, the effluent pH did not change significantly during reactor operation (two tailed t -test, $p = 0.07$) and averaged 6.9 ± 0.3 for days 81–217. When acetate was used as the electron donor (days 81–114), the average influent NO_3^- -N concentration was $15.4 \pm 0.6 \text{ mg L}^{-1}$ and effluent NO_3^- -N concentrations were below the method detection limit ($0.05 \text{ mg L}^{-1} \text{ N}$), excluding three data points (days 101, 102 and 114) (Fig. 2B). For the same period, the influent perchlorate concentration averaged $104.4 \pm 4.9 \text{ mg L}^{-1}$ and perchlorate was reduced to $0.22 \pm 0.13 \text{ mg L}^{-1}$ in the effluent (Fig. 2A). The redox potential in the effluent averaged $-326 \pm 79 \text{ mV}$ (Fig. 2D), indicating that redox conditions favorable for biological perchlorate reduction were established.³⁷ Influent COD concentrations were maintained at $199.8 \pm 11.9 \text{ mg L}^{-1}$ during operation

with acetate. Acetate COD concentrations in the effluent averaged $18.09 \pm 8.02 \text{ mg L}^{-1}$, which closely matched effluent sCOD concentrations ($17.8 \pm 10.2 \text{ mg L}^{-1}$).

On day 114, acetate was replaced with the glycerol product dosed at a target COD value equal to the acetate COD dose applied for days 81–114. This change in electron donor composition briefly impacted nitrate removal (Fig. 3), but effluent nitrate concentrations fell below detection again during days 116–142. Perchlorate removal also was impacted by the change in electron donor and effluent perchlorate concentrations first increased (91.5 mg L^{-1} and $84.8 \text{ mg L}^{-1} \text{ ClO}_4^-$ on days 115 and 116, respectively), decreased briefly (11.2 mg L^{-1} and 8.5 mg L^{-1} on days 119 and 123) and then increased gradually ($54.3 \pm 10.0 \text{ mg L}^{-1}$ during days 127–142). The higher effluent perchlorate levels for days 127–142 corresponded with higher ORP (Fig. 2D) and lower effluent sCOD ($4.7 \text{ mg L}^{-1} \pm 2.2 \text{ mg L}^{-1}$) (Fig. 3A).

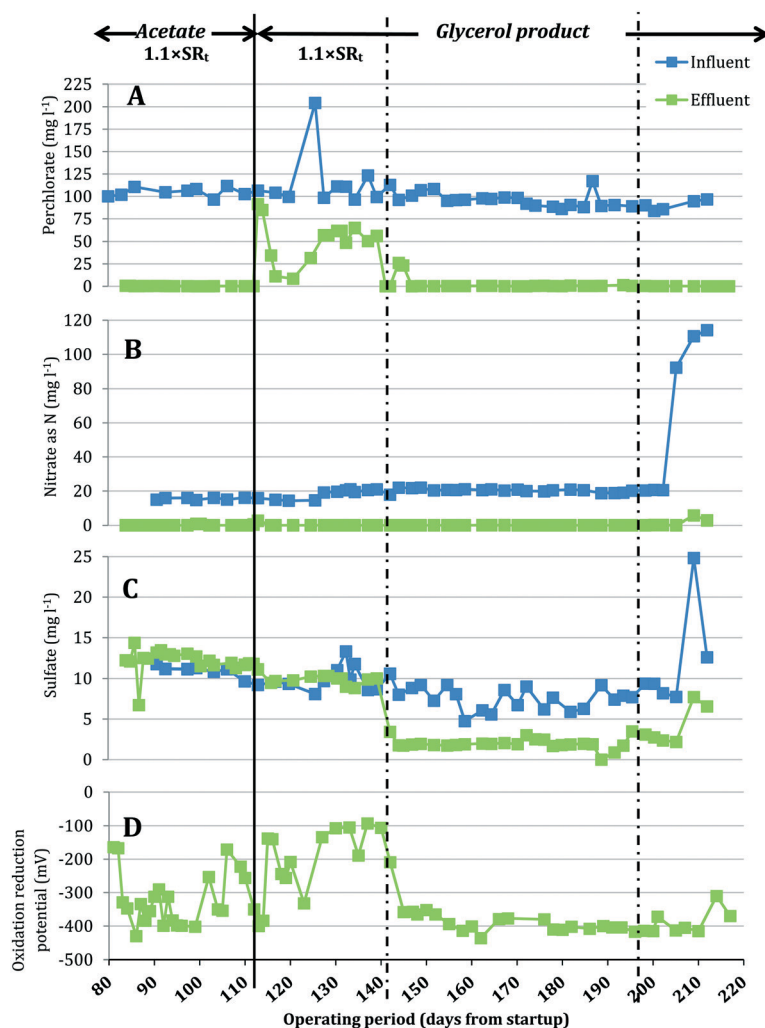


Fig. 2 Influent and effluent concentrations of (A) perchlorate, (B) nitrate, and (C) sulfate, and (D) effluent oxidation reduction potential in the FBR after a start-up period of 80 days (data for days 0–80 are not shown). During days 81–114, influent acetate COD was fed at $1.1\times$ the theoretical stoichiometric requirement (SR_t) for removal of $15 \text{ mg L}^{-1} \text{ NO}_3^-$ -N, $100 \text{ mg L}^{-1} \text{ ClO}_4^-$, and $39 \text{ mg L}^{-1} \text{ DO}$ (including $7 \text{ mg L}^{-1} \text{ DO}$ in the influent and $32 \text{ mg L}^{-1} \text{ DO}$ resulting from the complete reduction of $100 \text{ mg L}^{-1} \text{ ClO}_4^-$). On day 114, acetate was replaced by the glycerol product dosed at a target of $1.1\times \text{SR}_t$. Subsequently, influent COD of the glycerol product was increased on day 142 and then decreased on day 197. A reactor maintenance error resulted in unusually high influent nitrate and sulfate levels after day 200.



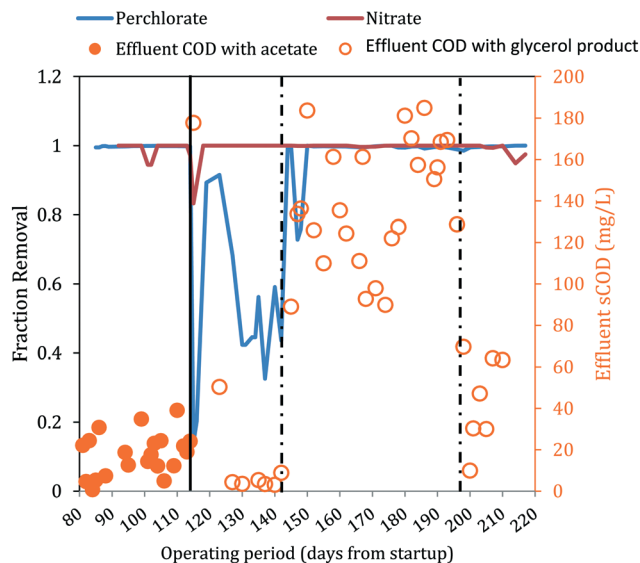


Fig. 3 Fraction removal of perchlorate and nitrate and effluent sCOD concentrations. The solid line indicates when acetate was changed to a glycerol electron donor. Influent COD levels were adjusted twice (represented by dashed lines). Closed circles are effluent acetate COD. Open circles are effluent sCOD measured either directly by COD analysis or indirectly through a correlation with effluent sNPOC measurements (ESI†).

After the influent concentration of the glycerol-based electron donor was increased on day 142 (influent COD was measured to be 484.19 mg L^{-1} on day 142), the effluent perchlorate concentrations decreased to an average of $0.28 \pm 0.20 \text{ mg L}^{-1}$ for days 145–193, excluding two measurements (26.1 mg L^{-1} and 23.2 mg L^{-1} on days 147 and 148, respectively). The effluent nitrate concentrations were below the detection limit during this period except on days 186, 189 and 193 when the effluent had 0.11 , 0.13 and $5.65 \text{ mg L}^{-1} \text{ NO}_3^- \text{-N}$, respectively. While the higher influent COD helped to restore perchlorate removal, significant sulfate reduction ($73 \pm 9\%$ sulfate removed) was observed during days 145–193 (Fig. 2C). Lowering the influent COD on day 197 to approximately 442 mg L^{-1} helped to lessen sulfate reduction and the effluent sulfate concentration increased from $1.94 \pm 0.62 \text{ mg L}^{-1} \text{ SO}_4^{2-}$ (days 145–193) to $2.77 \pm 0.53 \text{ mg L}^{-1}$ (days 200–210), but sulfate reduction was not completely eliminated. Lowering the influent COD did not affect perchlorate removal (*i.e.*, effluent perchlorate concentrations averaged $0.26 \pm 0.10 \text{ mg L}^{-1}$ for days 200–210).

3.2 Biomass yield with the glycerol-based electron donor

More biomass production was observed visually within 24 hours after changing from acetate to the glycerol-based electron donor and the reactor required more frequent wasting of biomass to prevent clogging and control bed expansion. Estimates of biomass yield indicated that the yield was higher with the glycerol product ($0.54 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{substrate}}$) than with acetate ($0.34 \text{ g COD}_{\text{biomass}} \text{ g}^{-1}$

$\text{COD}_{\text{substrate}}$) even when influent COD levels were similar (Table 2). The estimated biomass yield increased further ($0.62 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{substrate}}$) after the influent concentration of the glycerol product was increased on day 142. This increased yield observed with the glycerol product is consistent with previous reports of higher biomass yields with glycerol compared with other electron donors. In a bench-scale study with denitrifying, moving bed biofilm reactors, biomass growth was observed to be thicker and heavier with glycerol as the electron donor compared with methanol or sulfide.³⁸ This study also reported that reactor operation with glycerol was associated with higher levels of effluent total suspended solids and greater plugging of media void spaces.³⁸ Their estimate of the observed biomass yield with a glycerol-based electron donor ($0.5 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{substrate}}$ [based on average effluent volatile suspended solids data]) is close to the estimated yield with the glycerol product tested in the present study ($0.54 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{substrate}}$, Table 2). Similarly, in a study with bench-scale, denitrifying sequencing batch reactors, the biomass yield increased over time as biomass grew on glycerol or a glycerol by-product from biodiesel fuel production.²⁵

Microbial populations that use glycerol with higher yields likely increased their relative abundance in the FBR after the change in electron donor, as suggested by Al-Omari *et al.*³⁹ With more electrons channeled to biomass production, fewer electrons would have been available for the reduction of the electron acceptors. Indeed, replacing acetate with the glycerol product at an equivalent COD negatively affected perchlorate removal from days 130 to 142 (Fig. 3), during which time relatively low levels of soluble COD were also observed in the effluent ($4.8 \pm 2.4 \text{ mg L}^{-1}$ sCOD) (Fig. 3A). As discussed above, after the influent concentration of the glycerol-based electron donor was increased on day 142, the effluent perchlorate concentrations decreased. Therefore, the changing effluent perchlorate levels observed between days 115 and 143 after converting the electron donor from acetate to the glycerol product was likely caused by substrate-limitation brought on by a higher biomass yield with the glycerol product.

3.3 Bacterial community structure

To determine the microbial community structures in the FBR under two electron donor regimes, 16S rRNA genes extracted from biomass collected during operation with acetate or MicroC2000™ were sequenced using 454 pyrosequencing. Additionally, 16S rRNA genes from biomass collected from another bench-scale FBR and a fixed-bed system (Fig. S1†) fed $200 \mu\text{g L}^{-1} \text{ ClO}_4^-$ and $15 \text{ mg L}^{-1} \text{ NO}_3^- \text{-N}$ were sequenced. These “low-perchlorate” systems were operated using acetate as the electron donor for 100 days and then acetate was replaced with MicroC4000™, a carbohydrate-based, proprietary product with unknown composition. We compared the community structures in the low-perchlorate systems with the community structure in the high-perchlorate FBR to discuss



Table 2 Estimated biomass yields for four different electron donor operating conditions distinguished by composition or influent COD concentration. Acetate was replaced with the glycerol-based product on day 114. Yield was estimated based on COD consumed and the calculated COD requirement for the amount of perchlorate, nitrate and DO removal observed (sulfate reduction was included for operating conditions 3 and 4 only). Table S2 provides additional details and yield determinations for other time periods

Operating condition	Period reported (days)	Electron donor	Measured influent COD (mg L ⁻¹)	Period included for yield calculations (days)	Estimated yield ^a (mg COD _{biomass} mg ⁻¹ COD _{electron donor})
1	81 to 114	Acetate	200	102 to 114	0.34
2	115 to 142	Glycerol product	216	130 to 142	0.55
3	143 to 197	Glycerol product	484	178 to 189	0.61
4	197 to 222	Glycerol product	442	200 to 210	0.69

^a COD consumed beyond the amount required for contaminant removal was considered to be used for biomass growth. Yield was estimated as the ratio of COD used for biomass growth to total COD consumed. COD equivalents of 2.86 mg COD mg⁻¹ NO₃⁻-N, 0.67 mg COD mg⁻¹ ClO₄⁻, and 0.322 mg COD mg⁻¹ SO₄²⁻ were used in the calculations.

the impact of bulk electron acceptor concentrations and reactor configurations.

Bacterial members of 15 phyla were present in the three reactors, and *Proteobacteria* were most abundant (Fig. S3†). Perchlorate-reducing bacteria that perform canonical perchlorate respiration have been isolated exclusively from *Proteobacteria*.⁴⁰ *Dechloromonas* and *Azospira*, two genera within the *Betaproteobacteria*, encompass the (per)chlorate-reducing species most commonly isolated from the environment.⁴⁰ The majority of the sequences associated with *Dechloromonas* in the high-perchlorate FBR were from a single OTU (*Dechloromonas*_OTU0003 in Fig. 5), which was also the dominant *Dechloromonas* OTU in the low-perchlorate FBR and fixed-bed systems, but to a lesser extent (Fig. 5). Many perchlorate-reducing bacteria can respire nitrate and *Dechloromonas* spp. have been found to be abundant in nitrate-reducing systems even in the absence of perchlorate,⁴¹ including in the nitrate- and arsenic-removing bioreactor,³⁰ which provided biomass to inoculate the FBR and the low-perchlorate reactors. Therefore, the presence of nitrate was likely important for the initial selection of *Dechloromonas* OTU0003, but the higher bulk perchlorate concentrations in the high-perchlorate FBR (e.g., approximately 250 μg L⁻¹ compared with 6 μg L⁻¹ ClO₄⁻ in the low-perchlorate FBR) apparently enriched for this population. In the high-perchlorate FBR, the relative abundance of *Dechloromonas* increased from 5.8% to 32.1% over the reactor operating period (Fig. 5). Enrichment of *Dechloromonas* species in communities fed high concentrations of perchlorate is consistent with findings from other studies. For example, *Dechloromonas* represented 69% of clones from a mixed culture acclimated to 50–1500 mg L⁻¹ ClO₄⁻.⁴² In addition, the dominant (per)chlorate-reducing bacteria in a sequence batch reactor treating 1200 mg L⁻¹ ClO₄⁻ included *Dechloromonas* (19% of clones), although *Dechlorosoma* (53%) and *Ideonella* (28%) species were more abundant.⁴³ The enrichment for a single *Dechloromonas* OTU in the high-perchlorate FBR, compared with the many more *Dechloromonas* populations identified in the low-perchlorate systems (Fig. 5), implies that higher perchlorate concentrations select for a single

perchlorate-reducing population. The increase in relative abundance of OTU0003 from 7.6% (day 96) to 19.2% (day 189) after the change from acetate to MicroC2000™ suggests that this population can utilize glycerol efficiently.

The perchlorate- and nitrate-reducing *Azospira suillum* JPLRND²⁹ was used as one of the inocula in this study. OTU0051 matched the 16S rRNA gene sequence of *Azospira suillum* PS⁴⁴ (Fig. S4†) and, therefore, it is possible that this *Azospira* population is the *Azospira suillum* JPLRND in the inoculum. This OTU was present in the first set of biomass samples collected from all reactors (day 86). However, it was not abundant in the FBR relative to other community members during operation with either acetate or the glycerol product (Fig. 5). The strain was originally isolated using medium amended with 1000 mg L⁻¹ ClO₄⁻, although the groundwater from which the strain was isolated contained only 300 μg L⁻¹ ClO₄⁻.⁴⁵ Apparently this strain was not able to compete with other perchlorate-reducing bacteria in the FBR. Further evidence of its competitive exclusion from the FBR is that the putative inoculum had higher relative abundances in fixed-bed and FBR systems with lower bulk perchlorate levels (Fig. 5) but inoculated in the same way.²⁷

Operation of the FBR with acetate for 114 days before converting to the glycerol-based product means that the final bacterial community structure was derived from the community that developed first with acetate. This sequence of electron donors was chosen to represent a practical scenario in which an electron donor commonly used for biological, inorganic contaminant removal (e.g., acetate) is replaced by an alternative, proprietary product. Thus, certain populations that were present during operation with the glycerol-based electron donor may have been present because they were initially established during operation with acetate. The abundance of *Zoogloea* spp., which can utilize nitrate as an electron acceptor⁴⁶ and have been found to be abundant in bioreactors fed acetate,⁴⁷ while the glycerol-based product was used may have been contingent on the use of acetate as the electron donor first. *Zoogloea* spp. were abundant in all three perchlorate reactors (16.0%, 6.1%, and 14.3% relative



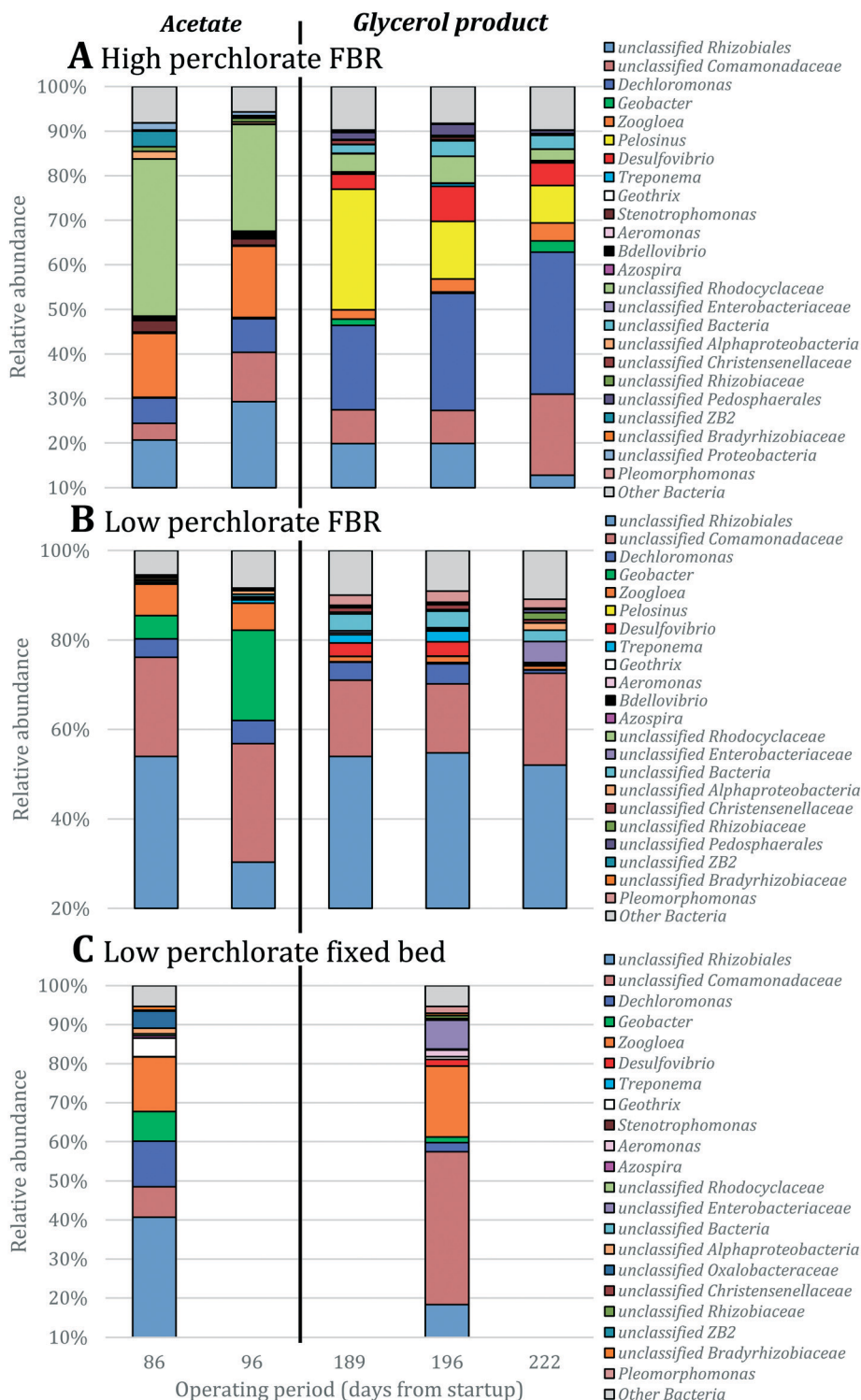


Fig. 4 Relative abundance of genera of the top 20 most abundant OTUs. Data were obtained for biomass collected from (A) the FBR and (B) an identical FBR and (C) a fixed-bed bioreactor fed $200 \mu\text{g L}^{-1} \text{ClO}_4^-$ as described previously.²⁷ The low-perchlorate systems were fed much lower concentrations of perchlorate and were operated using acetate or a proprietary carbohydrate-based electron donor. Truncated y axes are shown to accentuate changes in abundance.

abundance on day 96 in the high- and low-perchlorate FBRs and low-perchlorate fixed-bed reactor, respectively) but decreased markedly in the FBRs after replacing acetate with the glycerol-based product. In contrast, the relative

abundance of *Zoogloea* spp. increased slightly to 18.3% in the fixed-bed reactor after converting to the glycerol product. Compared with biofilms in a fixed-bed reactor, biofilms in an FBR are expected to have higher detachment



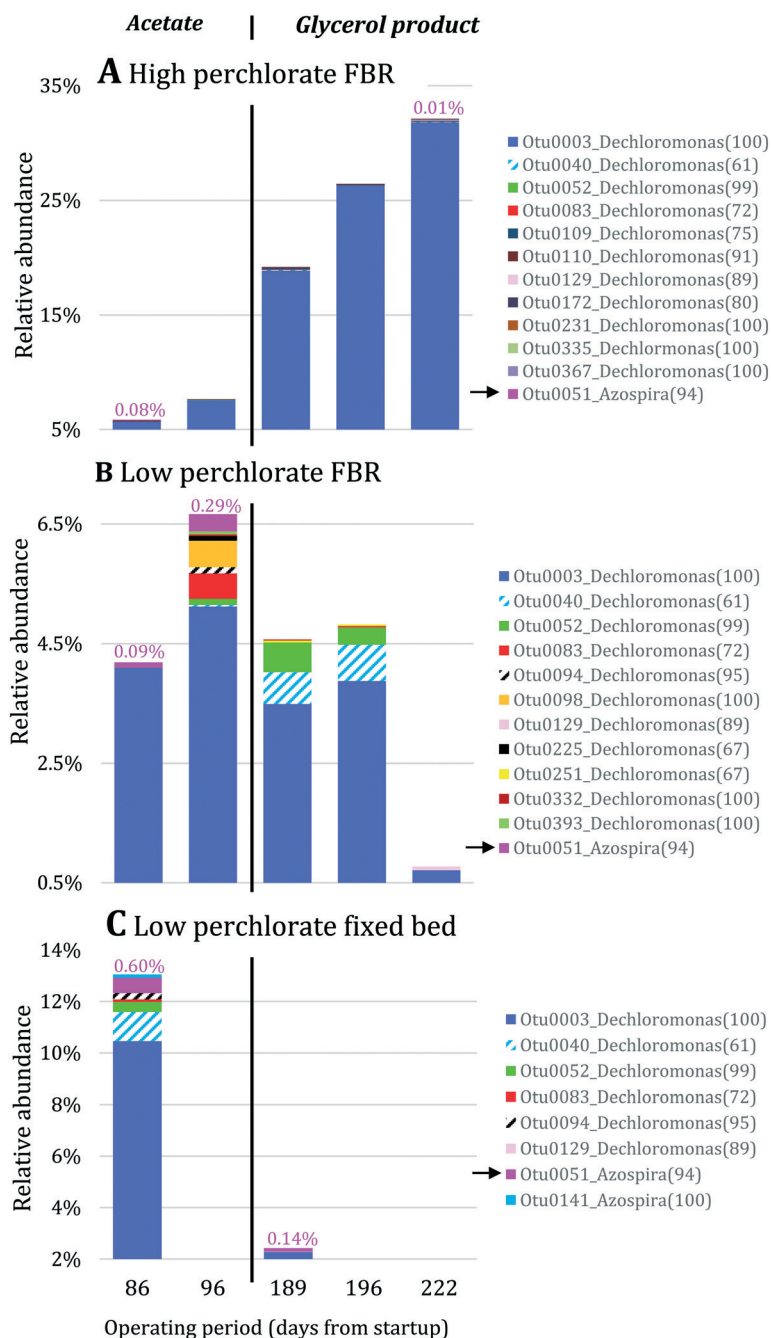


Fig. 5 Relative abundance of OTUs classified as *Dechloromonas* and *Azospira* based on 16S rRNA gene sequencing. Singleton OTUs are not included. Data were obtained for biomass collected from (A) the FBR, (B) a low-perchlorate FBR and (C) a low-perchlorate fixed-bed reactor. The low-perchlorate systems treated much lower concentrations of perchlorate (approximately $200 \mu\text{g L}^{-1}$) and were operated using acetate or a proprietary carbohydrate-based electron donor (Fig. S1†).²⁷ Arrows point to the putative *Azospira* inoculum seeded into the three reactors. Numbers above bars represent the percent relative abundance of the *Azospira* inoculum. Data were normalized by total number of 16S rRNA gene sequences. Samples from the FBRs were collected twice during reactor operation with acetate (days 86 and 96) and three times after converting acetate to the proprietary products (days 189, 196 and 222). Samples from the fixed-bed reactor were collected once before and once after converting to the carbohydrate-based product.

rates considering the particle-to-particle contact and turbulence that occurs with fluidization of the media,⁴⁸ and therefore there may be less retention of populations in biofilms in an FBR compared with biofilms in a fixed-bed system.

3.4 Nitrate utilization

Nitrate removal was impacted only briefly and to a lesser extent than perchlorate removal after the change in electron donor (Fig. 3A), suggesting that nitrate reduction was favored before perchlorate reduction. The preferential use of



nitrate before perchlorate is unexpected from a thermodynamic perspective because (per)chlorate reduction yields slightly more energy than nitrate reduction at standard conditions ($\Delta G^{\circ}_{\text{ClO}_4^-} = -801 \text{ kJ (mol acetate)}^{-1}$ and

$\Delta G^{\circ}_{\text{NO}_3^-} = -792 \text{ kJ (mol acetate)}^{-1}$).^{15,49} However, the preferential

use of nitrate has been reported for some perchlorate-reducing cultures,²⁸ including *Azospira suillum* JPLRND, which was used to inoculate the FBR, and several undefined or environmental communities.^{27,50–56} Indeed, nitrate inhibition of biological perchlorate reduction has been reported in several studies.^{28,51,54,57–59} Understanding the utilization of these competing electron acceptors is important to optimize bioreactor operation and predict perchlorate reduction rates.

A few possibilities may explain why nitrate reduction was favored over perchlorate reduction in the FBR. Firstly, a single *Dechloromonas* population (*Dechloromonas*_OTU0003) increased in relative abundance over time and became the most abundant population in the reactor by the end of the study (Fig. 5A). This population may have been involved in reducing nitrate as well as perchlorate. The mixed community inoculum originated from a nitrate- and arsenic-removing bioreactor,³⁰ which was itself inoculated with biomass from a perchlorate- and nitrate-reducing bioreactor.²⁰ This history of nitrate-reducing conditions may have increased the selective pressure for *Dechloromonas* populations capable of respiring both nitrate and perchlorate. Nitrate reduction has also been attributed to *Dechloromonas* in other mixed community studies. For example, in a bench-scale, two-stage membrane biofilm reactor system, *Dechloromonas* dominated the bacterial community in the first reactor where the majority of nitrate reduction occurred.⁶⁰ If the dominating *Dechloromonas* (OTU0003) population in the FBR was responsible for the majority of nitrate and perchlorate reduction, it is unclear why this population would prefer nitrate reduction when the electron donor substrate was limited. While there are some examples of purified perchlorate reductase enzymes that reduce nitrate,⁶¹ many (per)chlorate-reducing bacteria that respire nitrate contain a separate nitrate reductase for nitrate reduction.^{62–65} It is therefore unlikely that nitrate competes with perchlorate for a single active binding site in perchlorate reductase, but without further experiments on the dominating *Dechloromonas* population in the FBR, it cannot be ruled out that the preferential use of nitrate before perchlorate observed was not related to competitive inhibition.

The presence of nitrate has been shown to significantly alter the community structure of perchlorate-reducing communities. In long-term, perchlorate-reducing enrichment cultures receiving acetate as the sole electron donor and seeded with marine sediment, *Rhodobacteraceae* were the prevailing populations based on shotgun metagenomics and 16S rRNA gene sequencing.⁶⁶ When nitrate was added in addition to perchlorate, the abundance of *Rhodocyclaceae* increased and *Rhodobacteraceae* decreased.⁶⁶ *Rhodocyclaceae* were well represented in the high-perchlorate FBR, particularly during op-

eration with acetate (Fig. 4), whereas *Rhodobacteraceae* were rare. More research is needed to understand if *Rhodocyclaceae* prefer nitrate over perchlorate. A selective pressure by the presence of nitrate could explain why nitrate removal was favored over perchlorate removal when acetate became limiting in low-perchlorate fixed-bed and recirculating bioreactors fed $1 \text{ mg L}^{-1} \text{ ClO}_4^-$ and $10\text{--}16 \text{ mg L}^{-1} \text{ NO}_3^- \text{ N}$, even though reactor biofilms had been enriched under perchlorate-reducing conditions.⁵¹ The presence of nitrate, irrespective of inoculation strategy, may have selected for microbial populations in the FBR that prefer nitrate reduction and/or compete effectively against perchlorate-reducing bacteria for space in the biofilm.⁶⁷

3.5 Sulfate reduction

While the higher influent COD after day 142 helped to restore perchlorate removal, significant sulfate reduction (73 ± 9% sulfate removed) was observed during days 145–193. The highest relative abundance of the sulfate-reducing bacteria *Desulfovibrio* observed in the high-perchlorate FBR (8.0% on day 196) corresponded with a period when sulfate reduction was high (88.0% of influent sulfate was reduced on day 196). Lowering the influent COD on day 197 to approximately 442 mg L^{-1} helped minimize sulfate reduction and the effluent sulfate concentration increased from $1.94 \pm 0.62 \text{ mg L}^{-1} \text{ SO}_4^{2-}$ (days 145–193) to $2.77 \pm 0.53 \text{ mg L}^{-1}$ (days 200–210), but sulfate reduction was not completely eliminated. Lowering the influent COD did not affect perchlorate removal (*i.e.*, effluent perchlorate concentrations averaged $0.26 \pm 0.10 \text{ mg L}^{-1}$ for days 200–210), and as the relative abundance of perchlorate-reducing *Dechloromonas* increased, so did the relative abundance of sulfate-reducing *Desulfovibrio* (Fig. 4). Similarly, in a membrane biofilm reactor, the greatest perchlorate removal was achieved when the greatest sulfate reduction occurred.⁵⁰ When influent COD from the glycerol product was decreased on day 197 and sulfate reduction subsequently declined, perchlorate removal did not change dramatically. This is in contrast with observations in previous studies^{27,50} that reported a trade-off between perchlorate removal and the control of sulfate reduction. In the study by Ontiveros-Valencia and colleagues (2013), reducing the electron donor concentration in the influent fed to a membrane biofilm reactor not only helped control sulfate reduction but also resulted in slightly higher effluent perchlorate concentrations.⁵⁰ They suggested that sulfate reducers compete with perchlorate reducers for space in the biofilm.⁵⁰

3.6 Incomplete perchlorate reduction

Even with greater than 99.5 percent removal of perchlorate in the FBR, average effluent perchlorate concentrations during steady-state operation with acetate and the glycerol product were still well above the U.S. EPA's recommended preliminary remediation goal of $15 \mu\text{g L}^{-1} \text{ ClO}_4^-$ for Superfund sites with a drinking water exposure pathway. Given the concentrations



of sCOD in the effluent during the same time periods (Fig. 3), the incomplete removal of perchlorate in the FBR was probably not caused by electron donor substrate limitation. We speculate that even within the biofilm, the electron donor substrate concentration was not limiting perchlorate reduction as the biofilm in an FBR is typically thin due to increased detachment caused by particle-to-particle attrition and turbulence⁴⁸ and thus did not promote concentration differences between the bulk liquid and the areas within the biofilm close to the substratum. Biofilm modeling could be used to evaluate possible substrate limitations but is beyond the scope of the current study. In addition, the relative abundance of *Dechloromonas* species increased during the reactor operating period and, by the end of the study, comprised greater than 30% of the bacterial community (Fig. 4 and 5), suggesting that incomplete perchlorate reduction was not caused by low abundance of perchlorate-reducing populations in the system. We recognize that the relative abundance of perchlorate-reducing bacteria based on sequencing of 16S rRNA genes may not provide an accurate representation of perchlorate-reducing activity. Quantifying the expression of functional genes related to perchlorate reduction (e.g., chlorite dismutase or perchlorate reductase^{68,69}) or sequencing of 16S rRNA⁷⁰ would have provided additional information on the activity of perchlorate-reducing species and their involvement in perchlorate removal.

One reason the FBR did not remove perchlorate to lower levels in the effluent may be explained by considering characteristics of steady-state biofilms. To sustain a steady-state biofilm, the bulk electron acceptor substrate concentration must be above a minimum concentration (S_{\min} , eqn (1)), so that growth occurs rapidly enough to replace biofilm losses due to cell decay (b_{decay} is a decay rate, d^{-1}) and detachment (b_{detach} is a detachment rate, d^{-1}).⁷¹

$$S_{\min} = \frac{K_s (b_{\text{decay}} + b_{\text{detach}})}{\mu_{\max} - b_{\text{decay}} - b_{\text{detach}}} \quad (1)$$

A perchlorate concentration below S_{\min} is expected to lead to diminishing biofilm thickness and wash-out of perchlorate-reducing populations. S_{\min} changes proportionally with the half-saturation constant (K_s), the concentration of electron acceptor substrate at which a population grows at half its maximum growth rate (μ_{\max}), and K_s varies inversely with a population's affinity for the electron acceptor substrate. Systems with low concentrations of perchlorate are expected to select for perchlorate-reducing populations with higher affinity (and lower K_s values) for perchlorate and/or populations that utilize perchlorate as a secondary growth substrate,⁷² whereas systems with high perchlorate concentrations would select for populations which use perchlorate as a primary substrate and have lower affinity for perchlorate.⁷³ Interestingly, despite the two orders of magnitude difference in bulk perchlorate levels between the high and low perchlorate systems, the same *Dechloromonas*

population (OTU0003) dominated the bacterial community in all three bioreactors (Fig. 5). While several more *Dechloromonas* populations were abundant in the FBR with low perchlorate (Fig. 4 and 5), OTU0003 *Dechloromonas* was particularly dominant in the high-perchlorate FBR and presumably outcompeted the other *Dechloromonas* populations in this system. It is possible that higher concentrations of perchlorate selected for a *Dechloromonas* population with a lower affinity and higher K_s , thereby driving up the lowest perchlorate concentration attainable in the high-perchlorate FBR.

Detachment rate (b_{detach}) also may have limited the lowest perchlorate concentration achievable in the FBR. As discussed above, biofilms in an FBR are expected to have higher detachment rates than biofilms in fixed-bed systems considering the particle-to-particle contact and turbulence that occurs with fluidization of the media.⁴⁸ A higher detachment rate would result in higher effluent perchlorate concentrations (eqn (1)). In agreement with this, effluent perchlorate concentrations in the low-perchlorate FBR were higher (approximately $6 \mu\text{g L}^{-1} \text{ClO}_4^-$ for the first 100 days of reactor operation) than effluent concentrations in the fixed-bed reactor (less than $3 \mu\text{g L}^{-1} \text{ClO}_4^-$) treating the same influent for the same time period.²⁷

In a fixed-bed system, the influent moves through the bed in a plug-flow manner and concentration gradients occur along the depth of the bed.⁴⁷ Changes in electron acceptor concentrations along the depth of the fixed bed may allow greater spatial separation and selection for perchlorate-reducing bacterial populations with a wider range of affinities (K_s) for perchlorate. Consistent with this, a comparison of *Dechloromonas* populations in the fixed-bed system and in the low-perchlorate FBR (Fig. 5C) for the first biomass sampling event (day 86) revealed that several more *Dechloromonas* populations were present in the fixed-bed system. It is therefore possible that lower effluent perchlorate concentrations would have been achieved if a fixed-bed system would have been used to treat synthetic groundwater with the high perchlorate concentration. In practice, the mixing of microorganisms that occurs during backwashing reduces the spatial separation achieved during operation of a fixed-bed system.⁷⁴ Therefore, the use of a two-stage system with two bioreactors in series³⁰ may be a more promising approach as different microbial communities are expected to be selected for in the "lead" and "lag" reactors.^{47,60,75}

4. Conclusions and future directions

This study demonstrated the feasibility of replacing acetate with MicroC2000™, a proprietary glycerol-based electron donor, for biological reduction of 100 mg L^{-1} perchlorate from groundwater. Electron donor choice by the water treatment industry is determined primarily by cost and operational requirements. The need for higher COD doses and more frequent wasting of biomass with MicroC2000™



compared with acetate is consistent with our results of an earlier study testing MicroC4000™ for biological treatment of lower concentrations of perchlorate.²⁷ These observations with MicroC products require follow-up investigation through pilot and full-scale applications. More feasibility studies of diverse electron donors will provide the water treatment industry with greater flexibility for electron donor choice. A recent lifecycle assessment reported that the environmental impacts associated with the production of acetate contributed 87–98% of the overall environmental impacts of biological perchlorate reduction.⁷⁶ Future research investigating environmental impacts and life cycle costs associated with electron donors used for perchlorate reduction would provide additional information to guide electron donor choice. However, using a product with undisclosed composition makes it difficult to fully assess the product's environmental impacts. As several full-scale wastewater treatment plants are already using MicroC2000™ for denitrification, it appears that the use of MicroC2000™ has been accepted by the water treatment industry in spite of its undisclosed composition. As treated wastewater is increasingly being considered for water reuse applications, the use of proprietary products without full disclosure may need to be reconsidered.

Effluent perchlorate concentrations did not satisfy the U. S. EPA's recommended preliminary remediation goal for Superfund sites. We hypothesize that the incomplete perchlorate removal observed was partly because high perchlorate loadings enriched for a single *Dechloromonas* population with low affinity for perchlorate. Utilizing two FBRs in series may achieve better effluent quality by supporting the growth of perchlorate-reducing populations with higher affinity for perchlorate in the second reactor. However, this two-stage approach would increase the physical and energy footprints of treatment. More research on community structure along concentration gradients that develop in fixed-bed systems, and the effects of routine backwashing on the positioning of microbial populations in fixed-bed biofilms, is needed to evaluate whether a single fixed-bed bioreactor can be competitive with a two-stage FBR in terms of effluent quality and operational convenience.

Acknowledgements

This work was partially funded by Environmental Operating Solutions, Inc (EOSi). While EOSi was a partner in this research, University of Michigan researchers executed the work, analyzed and interpreted all results and wrote the manuscript. NK was partially supported by a fellowship from the Susan E. Stutz-McDonald Scholarship Foundation. The authors would like to thank Ashley Hammerbeck and Xinsheng Chu for their help with reactor maintenance and chemical analyses, Paul Hatzinger and Shaw Environmental for providing the *Azospira suillum* JPLRND culture, and Sam Ledwell for helpful discussions.

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