Pyrosequencing analysis yields comprehensive assessment of microbial communities in pilot-scale two-stage Membrane Biofilm Reactors

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Abstract

We studied the microbial community structure of pilot two-stage Membrane Biofilm Reactors (MBfRs) designed to reduce nitrate (NO$_3^-$) and perchlorate (ClO$_4^-$) in contaminated groundwater. The groundwater also contained oxygen (O$_2$) and sulfate (SO$_4^{2-}$), which became important electron sinks that affected the NO$_3^-$ and ClO$_4^-$ removal rates. Using pyrosequencing, we elucidated how important phylotypes of each “primary” microbial group – denitrifying bacteria (DB), perchlorate-reducing bacteria (PRB), and sulfate-reducing bacteria (SRB) – responded to changes in electron-acceptor loading. UniFrac, principal coordinate analysis (PCoA), and diversity analyses documented that the microbial community of biofilms sampled when the MBfRs had a high acceptor loading were phylogenetically distant from and less diverse than the microbial community of biofilm samples with lower acceptor loadings. Diminished acceptor loading led to SO$_4^{2-}$ reduction in the lag MBfR, and this allowed Desulfovibrionales (an SRB) and Thiothrichales (sulfur-oxidizers) to thrive through S cycling. Due to this cooperative relationship, they competed effectively with DB/PRB phylotypes such as Xanthomonadales and Rhodobacterales. Thus, pyrosequencing illustrated that, while DB, PRB, and SRB responded predictably to changes in acceptor loading, a decrease in total acceptor loading led to important shifts within the “primary” groups, the onset of other members (e.g. Thiothrichales), and overall greater diversity.

Keywords: pilot MBfR, nitrate, perchlorate, sulfate, pyrosequencing (deep sequencing), community structure, community function.
Introduction

Nitrate (NO$_3^-$) is a prevalent water contaminant due to its heavy use in fertilizers and widespread presence in wastewater. NO$_3^-$ can cause methemoglobinemia$^{1,2}$ in infants and spur eutrophication in water bodies. NO$_3^-$ is regulated by the US EPA,$^3$ which established a maximum contaminant level (MCL) of 10 mg N/L for drinking water.

Perchlorate (ClO$_4^-$) is an oxyanion with great chemical stability and is a constituent of rocket propellants, fireworks, and explosives. ClO$_4^-$, a normally recalcitrant contaminant found in waters of 35 US states and Puerto Rico,$^4$ can disrupt the thyroid after ingestion. Although ClO$_4^-$ is not yet listed as a regulated chemical,$^5$ the USEPA is planning to issue an MCL.$^6$ NO$_3^-$ and ClO$_4^-$ often are found together at contaminated sites, because ammonium nitrate (NH$_4$NO$_3$), ammonium perchlorate (NH$_4$ClO$_4$), and potassium nitrate (KNO$_3$) are used together for the production of rocket fuel and explosives.$^4$

Destruction of NO$_3^-$ and ClO$_4^-$ by microbial respiration has been well documented.$^7$-$10$ NO$_3^-$ reduction can enhance or hinder ClO$_4^-$ reduction$^{11}$-$14$ depending on the operating conditions of bioremediation approaches. Particularly, the inhibition of ClO$_4^-$ reduction originates from the competition between denitrifying bacteria (DB) and perchlorate-reducing bacteria (PRB) for common resources, such as the electron donor,$^{15}$ space in biofilms,$^{15}$ and reductase enzymes.$^{16}$-$18$ However and regardless of possible complications, simultaneous microbial respiration of NO$_3^-$ and ClO$_4^-$ has been reported.$^{19}$-$20$ Furthermore, the need to manage the microbial communities in the system becomes even more pressing when in addition to NO$_3^-$ and ClO$_4^-$, other electron acceptors such as sulfate (SO$_4^{2-}$) also are present in the water to be treated.
The presence of $\text{SO}_4^{2-}$ in a $\text{NO}_3^-$ and $\text{ClO}_4^-$-contaminated groundwater was the situation encountered during demonstration of a pilot two-stage Membrane Biofilm Reactor (MBfR) system. In the MBfR, hydrogen gas ($\text{H}_2$) diffuses through the walls of hollow-fiber membranes and is used as electron donor by microorganisms that grow as a biofilm on the membranes while utilizing oxidized compounds present in the water flowing through the reactor as electron acceptors. Previous research with MBfR biofilms pointed out competitive relationships between $\text{NO}_3^-$ and $\text{ClO}_4^-$ reductions for which a $\text{NO}_3^-$ loading above 0.6 g N/m$^2$ day at a fixed $\text{H}_2$-delivery capacity slowed $\text{ClO}_4^-$ reduction.

Based on the desire to minimize competition between $\text{NO}_3^-$ and $\text{ClO}_4^-$ reductions when the groundwater to be remediated had a high $\text{NO}_3^- : \text{ClO}_4^-$ ratio ($\sim$76 g N: 1 g $\text{ClO}_4^-$), Evans et al. set up a two-stage pilot-scale MBfR. The lead MBfR treated the raw groundwater and performed the bulk of denitrification. This lowered the $\text{NO}_3^-$ loading and the potential for $\text{NO}_3^-$ reduction to compete with $\text{ClO}_4^-$ reduction in the lag MBfR, which received the effluent from the lead MBfR. The strategy was mostly successful, since most of the $\text{NO}_3^-$ removal occurred in the lead MBfR; however, the two-stage pilot MBfR could not consistently drive the $\text{ClO}_4^-$ concentrations to below the detection limit of 4 µg/L.

In an initial effort to understand the pilot MBfR’s performance, Zhao et al. assessed the microbial community structure of the pilot reactors using the quantitative Polymerase Chain Reaction (qPCR) targeting characteristic reductases. DB (determined by the nitrite reductases $\text{nirK}$ and $\text{nirS}$) were the most abundant microbial group; however, sulfate-reducing bacteria (SRB) (quantified by the dissimilatory sulfite
reductase *dsrA*) became dominant and may have outnumbered DB in the pilot MBfRs when the NO$_3^-$ + O$_2$ loading was low, below 0.3 g H$_2$/m$^2$ day.$^{23}$ PRB (quantified by the perchlorate-reductase *pcrA*) were the smallest microbial fraction and were adversely affected when SRB became important, a finding consistent with previous bench-scale studies.$^{24}$

In contrast to these pilot results, Ontiveros-Valencia et al.$^{25}$ was able to achieve complete ClO$_4^-$ reduction in a two-stage bench-scale MBfR, even though the ClO$_4^-$ concentration was unusually high (~4000 µg/L) and SO$_4^{2-}$ was amply present (~55-60 mg/L). The success was attributed to an effective management of the microbial ecology of the reactors so that SO$_4^{2-}$ reduction was minimized, especially in the lag MBfR.

Ontiveros-Valencia et al.$^{25}$ suppressed SRB in the lag MBfR by re-oxygenating the influent to the lag MBfR to increase the total-acceptor loading and by lowering the H$_2$ availability by either decreasing the H$_2$ pressure or by using a less-H$_2$ permeable membrane. Neither strategy was followed with the pilot two-stage MBfR system: Re-oxygenation of the effluent from the lead MBfRs was not possible with the pilot configuration, and the pilot-MBfRs were mostly run with excess H$_2$ availability to encourage ClO$_4^-$ reduction.$^{21}$

Added to the fact that treatment is more challenging when SO$_4^{2-}$ is present in the water to be treated, only limited information is available on the ecological interactions between SRB and PRB. Waller$^{26}$ suggested that the microbial community structure of consortia explored in her study was responsible for the decline in ClO$_4^-$ reduction when high SO$_4^{2-}$ concentration was available. However, other studies reported no effect from
SO₄²⁻ on ClO₄⁻ microbial reduction²⁷-²⁸. Thus, more research addressing these critical ecologic interactions is needed.

Although Zhao et al.⁴ provided a broad view of the “primary” respiratory groups (i.e., DB, PRB, and SRB) in the pilot MBfRs, we employ high-throughput pyrosequencing to gain a deeper understanding of the microbial community structure, including more insight into the phylotypes that constitute the primary respiratory groups present when NO₃⁻, ClO₄⁻, and SO₄²⁻ are the electron acceptors and a view of other members within the biofilm.

Our study addresses the ecological interactions among DB, PRB, SRB, and other microbial groups that developed during bioremediation of groundwater polluted with NO₃⁻ and ClO₄⁻ with SO₄²⁻ also present. In particular, we use UniFrac and principal coordinate analysis (PCoA)²⁹,³⁰ to demonstrate that distinctly different communities developed in the biofilm when the acceptor-loading rate was decreased significantly. Furthermore, we explore how decreased acceptor loading led to shifts within the primary members and the development of important other members (e.g., heterotrophs and sulfur-oxidizing bacteria) in the community. While Zhao et al.⁴ used qPCR to provide an analysis of community structure according to the primary respiratory groups, our findings discriminate among conditions significantly altering the community structure, making the biofilm more diverse and causing shifts within and outside the primary microbial groups.


Materials and Methods

MBfR configuration and performance

Detailed information about the pilot-MBfRs configuration is given by Evans et al.\textsuperscript{21} and Zhao et al.\textsuperscript{23} In brief, the two-stage MBfR was composed of two 500-gallon (1890-L) vessels containing 4 MBfR modules with membrane surface area of 144 m\textsuperscript{2} per module. The manufacture and on-site configuration of the pilot-MBfR modules was done by APTwater and CDM-Smith. Figure 1a shows that the pilot-MBfR modules were cylindrical and made of woven fabric of polypropylene fibers, which formed sheets of fibers wrapped around a perforated acrylonitrile butadiene styrene (ABS) core. Each module contained ~140,000 polypropylene fibers (200\,µm OD, Teijin, LTD, Japan). H\textsubscript{2} gas diffused through the fiber sheet, and water passed through the perforations in the ABS core. The lead and lag MBfRs also were equipped with a set of side reactors for taking biofilm samples without disturbing the biofilm in the modules.\textsuperscript{21,23} Figure 1b&c shows the side reactors with their connections for water and H\textsubscript{2}.

The pilots were set up to treat a site historically used for munitions and explosives manufacture and surroundings agricultural fields. Hence, the oxidized contaminants in the groundwater were NO\textsubscript{3}\textsuperscript{−} at 8-9 mg N/L and ClO\textsubscript{4}\textsuperscript{2−} at 160-200 µg/L. The influent also contained O\textsubscript{2} at ~8 mg/L and SO\textsubscript{4}\textsuperscript{2−} at ~22 mg/L. The lead and lag positions were switched every 3 days to make the biofilm development similar in both MBfRs and with the goal of minimizing the abundance of SRB in the lag MBfR.\textsuperscript{21} The H\textsubscript{2} pressure and influent flow rate were adjusted according to the conditions in Table 1. The four conditions are representative periods of continuous operation of the pilot system. Adjustment of the influent flow rate led to a proportional change in the total electron-
acceptor surface loading: Conditions 3 and 4 had significantly lower total electron acceptor loadings than did Conditions 1 and 2. The use of an excess H₂-delivery capacity was done to ensure good NO₃⁻ removal in the lead MBfR and to achieve complete ClO₄⁻ reduction in the lag MBfR.²¹

Samples were collected for off-site analysis at Test America (Irvine, CA), which is certified by the California Environmental Laboratory Accreditation Program (ELAP). The off-site assessment involved measurements for the lead and lag concentrations of NO₃⁻ and SO₄²⁻ (US EPA method 300) and ClO₄⁻ (US EPA 314); they were performed three, one, and three times per week, respectively. In addition, measurements for NO₃⁻ and sulfide (as a surrogate for SO₄²⁻ reduction) were carried out three times per week on-site using field kits (CHEMetrics, Virginia, USA).²¹ O₂ and pH were measured by a hand held probes.²¹ The pH during operation was maintained between 7.4-7.8. The maximum H₂ delivery capacity was calculated according to Tang et al. ³¹ and reported in Table 1. Our work is complementary to the work reported by Zhao et al. ²³, and both studies are built on the field demonstration described by Evans et al.²¹

Side reactors representing conditions 1, 2, 3, and 4 were taken after 60, 116, 221, and 263 days of continuous operation, respectively, and were sent in ice containers to the Swette Center for Environmental Biotechnology for microbial community analysis. The samples arrived within 24 hours and were processed according to Zhao et al.²³ for DNA extraction. DNA samples were stored at -80°C until shipping for 454 pyrosequencing. DNA samples for 454 pyrosequencing were sent to the Molecular Research DNA lab (Austin, Texas, USA), which performed amplicon pyrosequencing using a standard
Roche 454/GS-FLX Titanium. The Bacteria domain was targeted by selecting the V6 and V7 regions of the 16S rRNA gene with primers 939F (5' -
TTGACGGGGGCCCGCAC -3') and 1492R (5'TACCTTGGTTACGACTT-3'). We processed the raw data using QIIME 1.7.0 suite and removed sequences having fewer than 250 bps, homopolymers of more than 6 bps, primer mismatches, or an average quality score lower than 25. We picked the operational taxonomic unit (OTUs) using the Greengenes 16S rDNA database with uclust based on ≥ 97% identity, removed OTUs that contain less than two sequences (singletons) from our analysis, and aligned the representative sequence of each OTU to the Greengenes Database using PyNast.

Potentially chimeric sequences were identified by using ChimeraSlayer, and a python script in QIIME was employed to remove the chimeric sequences. We assigned taxonomy to OTUs with BLAST using the SILVA database and constructed Newick-formatted phylogenetic trees using FasTree.

For the purpose of eliminating heterogeneity related to having different numbers of sequences among the samples, we sub-sampled the OTU table by randomly selecting ten different times the lowest number of sequences (6800) found among the samples. We then generated PCoA plots and Unweighted Pair Group Method Arithmetic Mean (UPGMA) plots using jack-knifed beta diversity.

We estimated the OTU richness by calculating Chao1, which determines the asymptote on an accumulative curve, predicting how many OTUs would be present if a high number of sequences had been collected, and the phylogenetic relationships by using phylogenetic diversity (PD), which estimates the cumulative branch lengths from random OTUs. To evaluate the microbial species diversity and evenness, we computed
the Shannon$^{43}$ and Simpson$^{44}$ indexes. A higher value for the Shannon index indicates greater microbial diversity, while a value for the Simpson metric near one shows an even distribution of bacterial groups within the sample. Sequence data sets are available at NCBI/Sequence Read Archive (SRA) under study with accession number SRP038958.
Results and Discussion

Microbial community function

Table 2 synthesizes the performance of the pilot-scale reactors. The lead MBfRs were responsible for ~99% of the O\textsubscript{2} respiration, 70-90% denitrification, and a small loss of ClO\textsubscript{4}\textsuperscript{-}.\textsuperscript{21,23} In the lead MBfRs, the NO\textsubscript{3}\textsuperscript{-} + O\textsubscript{2} flux was greater than ~0.34 g H\textsubscript{2}/m\textsuperscript{2}.day\textsuperscript{23} (Table 2), which completely suppressed SO\textsubscript{4}\textsuperscript{2-} reduction and is consistent with the bench-scale results of Ontiveros-Valencia et al.\textsuperscript{45} and modeling work by Tang et al.\textsuperscript{46}

Therefore, NO\textsubscript{3}\textsuperscript{-} and SO\textsubscript{4}\textsubscript{2-} were the dominant electron acceptors entering the lag MBfR, and the total acceptor surface loading to the lag MBfR was much lower than for the lead MBfR (Table 1). Although the objective of reducing the flow rate and total acceptor loading for Conditions 3 and 4 was to enhance ClO\textsubscript{4}\textsuperscript{-} removal in the lag MBfR, its major impact was to favor SO\textsubscript{4}\textsubscript{2-} reduction, an undesired outcome that led to lower ClO\textsubscript{4}\textsuperscript{-} removal fluxes in the lag MBfR (Table 2).

Electron-acceptor loading affects microbial diversity and structure

Table S1 shows all the values for the diversity and evenness metrics for the four conditions. Overall, Chao1, Shannon, and PD values show that the microbial diversity of biofilm samples from Conditions 3 and 4, which had a low acceptor loading (Table 1), was greater than from Conditions 1 and 2, which had a higher acceptor loading.

Consistent with the Chao1 results and based on the Simpson index, biofilm samples from Conditions 3 and 4 were more evenly distributed than those in Conditions 1 and 2.

Figure 2 shows the unweighted UniFrac analysis of the biofilm samples, which is based on the presence or absence of all the phylotypes within a sample. The biofilm
samples with high acceptor loading (Conditions 1 and 2) clearly formed a cluster (blue branch) distinct from the cluster of Conditions 3 and 4 (red branch). Thus, the large changes in acceptor loading between Conditions 2 and 3 led to very different microbial communities. Particularly for Conditions 1 and 2, the lead and lag biofilms were not significantly different due to the regular switching of positions.  

Figure 3 presents the unweighted PCoA plot, which reinforces the clustering found with the UniFrac analysis. The biofilm communities of Conditions 1 and 2 were close to each other along the PC1 vector, while those biofilm samples of Conditions 3 and 4 were distant. In an attempt to differentiate the driving force for the PC1 vector, we connect the removal fluxes for SO$_4^{2-}$ and ClO$_4^-$ (Table 2) with the community analysis by PCoA. Conditions 3 and 4 had importantly decreased average acceptor loadings (Table 1), and SO$_4^{2-}$ reduction increased significantly (Table 2). The PC1 vector correlates with increased SO$_4^{2-}$ reduction, particularly from Condition 2 to Condition 3. Hence, the microbial community structure was substantially modified when SO$_4^{2-}$ reduction became a more important electron sink, a trend also noted by Ontiveros-Valencia et al.  

Condition 2 was different from Conditions 1, 3, and 4 along the PC2 vector. This trend is most likely explained by the substantially higher ClO$_4^-$ flux for Condition 2, which is illustrated in Table 2.  

While the low electron acceptor loadings primarily shaped the microbial community, particularly by favoring SO$_4^{2-}$ reduction, operation time also allowed biomass buildup that may have contributed to structural changes in the biofilm communities. However, operational conditions, such as to the flow rate and hydraulic retention time (HRT), are directly connected to the electron acceptor loadings:
Decreased flow rate and the consequent higher HRT cause a lowered electron acceptor loading. Extra H\textsubscript{2} delivery capacity also can frame the community on its own; however, the excess capacity to deliver electron donor rates was similar across conditions, while the loading of electron acceptor was significantly modified.

**Taxonomic breakdown and shifts in the microbial community structure**

Figure 4 synthesizes the taxonomical break down at the order level of the most abundant phylotypes. Figure S1 also reports the ten most abundant phylotypes for all conditions at the genus level. Consistent with UniFrac and PCoA, the biofilm communities of the lead and lag MB\textsubrscript{R} were similar for each Condition. The brackets in the legend of Fig. 4 identify the known DB, PRB, SRB, and other types. The groupings show four important trends. First, ~86% of the taxonomic breakdown had microbial phylotypes most closely related to characterized DB and PRB for Condition 1, but these primary groups decreased for subsequent conditions, being only ~60% by Condition 4. Connecting this community trend to community function, DB and PRB phylotypes (reported by pyrosequencing in Figure 4) follow the same trend as the NO\textsubscript{3}\textsuperscript{-}, O\textsubscript{2}, and ClO\textsubscript{4}\textsuperscript{-} fluxes (Table 2).

Second, the decrease of microbial phylotypes most closely related to DB and PRB was accompanied by significant increases in microbial phylotypes most closely related to SRB: from <1\% in Condition 1 to ~13\% in Condition 4. The SRB trend by pyrosequencing is similar to the SRB trend noted by Zhao et al.\textsuperscript{23} using qPCR; however, the qPCR study found that SRB had become the largest primary group in Condition 4, followed by DB and PRB. It is possible that qPCR overestimated SRB, because some
DB harbor the *dsrA* gene.\(^{47}\) Regardless of the method employed, the key trend is that SRB became important with lower acceptor loading. As noted by Ontiveros-Valencia et al.,\(^{24}\) SRB become detrimental to PRB when they are able to occupy the most favorable zones in the biofilm (near the H\(_2\)-delivering substratum).\(^{46}\) Therefore, incomplete ClO\(_4^-\) reduction in the lag MBfR can be at least partially attributed to increased competition from SRB.

Third, lowered electron acceptor loadings leading to augmented SO\(_4^{2-}\) reduction (Conditions 3 and 4) boosted the sulfur-oxidizing *Thiotrichales* and the SRB *Desulfovibrionales*. This combination points towards a cooperative relationship based on active S cycling in which *Thiotrichales* oxidizes H\(_2\)S produced by SRB while respiring NO\(_3^-\) to ammonia (NH\(_4^+\)). Sulfide oxidation by *Thiotrichales* provided additional SO\(_4^{2-}\) for SRBs, probably allowing SRB to grow to higher proportions than what would be predicted from the one-time reduction of SO\(_4^{2-}\). Figure S1 shows that closely related *Thiothix* phylotypes, which belong to the *Thiotrichales* order, were abundant at Conditions 3 and 4, and they might have imposed a risk for fouling the membranes due to its filamentous growth.\(^{49}\) *Thiothrix* can accumulate S granules in its interior from the oxidation of H\(_2\)S and form rosettes, which are arrangements of filaments.\(^{50-51}\) Staff operating the pilot MBfRs reported observing filaments in some biofilms. Sulfide oxidizers also were reported in MBfR biofilms by Zhao et al.,\(^{52}\) who observed abundant *Campylobacteriales* (sulfur-oxidizing bacteria), and by Ontiveros-Valencia et al.,\(^{25}\) who reported significant presence of *Ignavibacteriales* (green sulfur-oxidizing bacteria) and *Thiobacteriales* (sulfur-oxidizing bacteria) when SO\(_4^{2-}\) reduction was favored in bench-scale MBfRs. The differences in the phylotypes of the sulfur-oxidizers observed in the
bench- versus pilot-scale MBfRs probably can be attributed to the different inocula in each study. Despite the different inocula, the cooperative relationship between SRB and sulfur-oxidizing bacteria seems to be common once SO$_4^{2-}$ reduction becomes important and seems to have accentuated an ecological advantage for SRB.

Besides sulfur-oxidizers, heterotrophic microorganisms such as *Bacteroidales* and *Flavobacteriales* increased in Conditions 3 and 4. The heterotrophs likely consumed soluble microbial products, whose rate of release increased with high rates of SO$_4^{2-}$ reduction.$^{33,45}$ Likewise, the relative abundance of “unclassified” bacteria and minor phylotypes (microbial groups at <1% abundance) (not shown in Figure 3) went from an average ~3% in Condition 1 to ~8% in Condition 4. The upswing of heterotrophs, unclassified bacteria, and minor phylotypes was the foundation for the increase in the microbial diversity with decreased acceptor loading (Table S1). The greater abundance of other groups and SRB certainly imposed more competition for space in the biofilm, forcing PRB to less favorable positions in the biofilm (zones more likely to detach).$^{24,46}$ Recently, Martin et al.$^{53}$ employed modeling to explain how increased detachment hindered MBfR performance. Thus, increasing diversity in the biofilm was correlated with poorer performance for ClO$_4^-$ reduction.

Fourth, the DB and PRB groups showed important shifts with acceptor loading. In Conditions 1 and 2, *Rhodobacterales* were dominant; however, the most abundant DB and PRB phylotypes shifted to *Xanthomonadales* and *Rhodocyclales* in Conditions 3 and 4. In particular, closely related *Aquimonas* phylotypes, which belong to the *Xanthomonadales* order, were common to all biofilm samples, remaining in the biofilm regardless of competition (Fig. S1). In contrast, *Rhodobacterales* declined dramatically.
in Conditions 3 and 4. Species *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* can reduce chlorate (ClO$_3^-$) to chlorite (ClO$_2^-$); however, no growth was associated with this metabolism.$^{34}$

Other substantial shifts in the phylotypes most closely related to DB and PRB were observable. While the DB and PRB phylotype *Rhizobiales* remained relatively constant across conditions, the phylotype *Hydrogenophilales* increased in Conditions 3 and 4. Lastly, phylotype *Burkholderiales* decreased abruptly while phylotype *Pseudomonadales* decreased slightly. These substantial shifts in the DB and PRB support that the biofilm communities were functionally redundant, which allowed different phylotypes to gain or lose prominence as acceptor loading changed without affecting denitrification performance.

In conclusion, pyrosequencing allowed us to comprehensively assess the microbial community diversity and structure of pilot MBfRs. UniFrac, and PCoA helped us understand the main drivers for the shifts in microbial structures. Biofilm communities developed with low total acceptor loading were more diverse and phylogenetic distant from communities with a higher acceptor loading. Primary members (i.e., DB, PRB, and SRB) overall tracked the reduction of the electron acceptors, but showed important shifts with acceptor loading. The DB/PRB phylotype *Rhodobacterales* was significantly abundant at high acceptor loading; however, the phylotype *Xanthomonadales* was overall the most dominant DB/PRB phylotype in all biofilm samples. *Desulfovibrionales* and *Thiothrichales* appeared together at low acceptor loadings and when SO$_4^{2-}$ reduction was strong, suggesting S cycling that corresponded to a slowing of the ClO$_4^-$-reduction rate. Likewise, heterotrophic bacteria became more
important with lower acceptor loading. The abundance of SRB and sulfur-oxidizing partners, as well as heterotrophs, likely accentuated competition for space and forced PRB to less favorable positions in the biofilm. Thus, the increase in diversity with low acceptor loading was due to the increases in SRB, sulfur-oxidizers, and heterotrophs, and it correlated with poorer performance in terms of ClO$_4^-$ reduction.

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Supporting Information

Alpha-diversity metrics and ten most abundant genera in biofilm samples across the four conditions in the pilot system. This information is available free of charge via the Internet at http://pubs.acs.org/
Table 1 Four Conditions identified H$_2$ availability (controlled by H$_2$ pressure) and electron-acceptor surface loadings (adjusted by influent flow rate) for lead and lag MBfRs

<table>
<thead>
<tr>
<th>Condition</th>
<th>Flow rate $m^3/d$</th>
<th>Hydraulic Retention Time hours</th>
<th>H$_2$ pressure atm</th>
<th>NO$_3^-$-N surface loading g H$_2$/m$^2$-d</th>
<th>O$_2$ surface loading g H$_2$/m$^2$-d</th>
<th>SO$_4^{2-}$ surface loading g H$_2$/m$^2$-d</th>
<th>ClO$_4^-$ surface loading g H$_2$/m$^2$-d</th>
<th>Total electron acceptor surface loading g H$_2$/m$^2$ day</th>
<th>Average electron acceptor loading g H$_2$/m$^2$ day</th>
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We calculated the electron acceptor loading rates according to:

\[
Loading = \frac{Q \times (S^o)}{A}
\]  

(eq. 1)

where $Q =$ volumetric flow rate (L/day), $A =$ membrane surface area (m$^2$), and $S^o$ is the influent concentration (g/L) for an electron acceptor. Each electron acceptor loading value was normalized to g H$_2$/m$^2$ day based on stoichiometric relationships described elsewhere.$^{15-23,25}$ Total electron-acceptor loading was calculated as the sum of the loadings for O$_2$, NO$_3^-$, ClO$_4^-$, and SO$_4^{2-}$. The average electron acceptor loading was calculated from the lead and lag total electron acceptor loadings at each condition. The lead and lag positions were switched every three days; therefore, an average estimate of the acceptor loading is valuable. The HRT was the same for each reactor regardless of the position.
Table 2 Electron acceptor and donor fluxes for lead and lag MBfRs for the four conditions tested over time

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nitrate flux g H₂/m² day</th>
<th>Oxygen flux g H₂/m² day</th>
<th>Sulfate flux g H₂/m² day</th>
<th>Perchlorate flux g H₂/m² day</th>
<th>Total H₂ experimental flux g H₂/m² day</th>
<th>Maximum H₂ flux g H₂/m² day</th>
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<tbody>
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The electron acceptor fluxes were reported elsewhere. The maximum H₂ flux was calculated as Tang et al. and the oversupply of H₂ corresponded to the maximum H₂ flux minus the total H₂ experimental flux.
Figure 1  a Pilot MBfR module which shows the ABS core and woven fabric. The water and H$_2$ flows are pointed by arrows. b&c show side reactors which were sent to ASU for community analysis. The side reactors were operated as the pilot MBfRs. b shows the water lines feeding the side reactors, and c visualizes the gas connections for the H$_2$ fed, and a closer look of the biofilm in the fiber sheet.
Figure 2  Clustering based on the unweighted UniFrac analyses. The branch length represents the distance between biofilm samples in UniFrac units, as indicated by the scale bar. The labels on each branch indicate the biofilm sample of either lead or lag MBfR at the four conditions applied to the reactors. The blue branch correspond to the reactors operated at high electron acceptor surface loadings (Conditions 1 and 2), while the red branch reflect the microbial community performing under low total electron acceptor surface loading (Conditions 3 and 4).
Figure 3 Principal Coordinate Analysis (PCoA) based on the unweighted UniFrac.
Figure 4  Microbial community structure in lead and lag MBfRs at the order level. The sum does not add up to 100% in all cases because phylotypes < 1% are not shown. The brackets in the legend group the orders according to known members of the noted metabolic groups. DB/PRB phylotypes are shown which hatched fills that clearly show a decline from Condition 1 to Condition 4. Some members of the “heterotrophic microorganisms,” are capable of denitrification under specific circumstances, such as when using acetate as electron donor and carbon source.48
References


