Denaturing gradient gel electrophoresis analysis of a methyl *tert*-butyl ether degrading culture applied in a membrane bioreactor

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Abstract
A membrane bioreactor (MBR) was operated for the removal of methyl *tert*-butyl ether (MTBE) from water. Although the reactor was seeded with several cultures acclimated to MTBE degradation, a long start-up time was observed. Monitoring of the reactor with denaturing gradient gel electrophoresis (DGGE) revealed a dramatic shift in the MBR culture from the original seed culture, indicating that the membrane had exerted a selective pressure on the culture. The MBR culture was found to be dominated almost entirely by *Sphingomonas*, belonging to the α–4 subclass of the α-Proteobacteria. Several unique properties of *Sphingomonas*, including their characteristic outer membrane containing glycosphingolipids, as well as their extreme adeptness for xenobiotic degradation are hypothesized to have aided in their selection in this bioreactor.

Keywords
biodegradation, DGGE, membrane bioreactor, MTBE, *Sphingomonas*

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is a gasoline additive which has achieved prevalent application as a fuel oxygenate for the reduction of automobile emissions. High water solubility of MTBE combined with general mishandling of fuels (spills, leaking underground storage tanks, etc.) has led to widespread contamination of drinking water supplies. Biological removal of MTBE is a hopeful treatment option.

Despite its initial recalcitrance, aerobic biodegradation of MTBE has now been widely observed. Because MTBE is a poor growth substrate, however, yield of MTBE degrading microorganisms has consistently been reported to be very low, usually about 0.1 mg VSS/mg MTBE (Salanitro *et al.*, 1994; Wilson *et al.*, 2000). As a result, biomass retention has become a key operating parameter for engineered MTBE removal systems. Wilson *et al.* (2000, 2001) found that the application of a polyethylene porous pot for the separation of hydraulic residence time (HRT) from the solids retention time (SRT) greatly enhanced reactor performance.

Complete biomass control is one the many advantages of a membrane bioreactor (MBR). MBRs are thus considered to be especially suited for slow-growing cultures. Because of this, as well as its compact size, mobility, and capability for high treatment flow rates, the MBR system is an ideal candidate for treatment of MTBE contaminated water. MBRs have already begun to be applied in the field and have met varied success. One study showed that a reseeding of the reactor with an alternative culture greatly improved the performance of the system (Steffan *et al.*, 2001).

Few studies have investigated the effect of membrane separation on the culture characteristics. Cicek *et al.* (1999) demonstrated several qualitative differences between the cultures of a membrane bioreactor and an activated sludge system operated in parallel. Several factors, such as high sheer stress and varying mass transfer conditions can affect the composition of the culture.

The aim of this study was to monitor and identify the community structure of a membrane bioreactor operated for the removal of MTBE from water. A molecular tool, denaturing gradient gel electrophoresis (DGGE) was used to identify and compare the MBR culture with the seed cultures, which were enriched in porous pot reactors under a quiescent flow regime.

METHODS

Operation of MBR

Influent MTBE was supplied to the reactor at 5 mg/L. Total flow to the reactor was 142 L/day with a 1-hour hydraulic retention time (HRT). The flow was composed mostly (>90%) of granular activated carbon (GAC) treated Cincinnati tap water, which was monitored regularly for free chlorine breakthrough (Hach model CN-70, Loveland, CO). The remaining flow consisted of a nutrient feed containing essential salts and vitamins (~2%) and a sodium carbonate buffer solution (~7%) to maintain a pH between 7.2 – 7.7 within the aeration tank. MTBE was supplied via the buffer feed using a Model 11 high precision syringe infusion pump (Harvard Apparatus, Inc., South Natick, MA) and a 5mL fixed needle syringe (Hamilton Co., Reno, NV).

The aeration tank of the MBR was 12L in total volume maintained at 6L by an adjustable float switch controlling the bulk water feed pump. Aeration was supplied by a coarse air bubble diffuser maintaining the dissolved oxygen at about 4 mg/L. An air-powered motor provided aeration tank mixing. Temperature was controlled with heat exchangers in both the recycle loop and aeration tank. An additional monitor within the aeration tank assisted in maintaining a temperature between 18-20^{o}C. Two additional pumps supplied the pressure required for the membrane filtration process.

The reactor was seeded with a mixture of cultures enriched on MTBE in combination with other oxygenated compounds which were characterized in Pruden *et al.* (in press). All MBR solids were retained with zero sludge wastage excluding minimal sampling for volatile suspended solids/ total suspended solids (VSS/TSS) measurements.

Membrane characteristics

A ceramic (aluminum oxide-titanium dioxide) tubular ultrafiltration membrane manufactured by TECH-SEP KERASEP^{TM}, module reference KO1-X, (Rhone Poulenc, France) was operated under cross-flow conditions in this study. The total surface area was 0.085 m^2 with a molecular cut-off of about 300,000 Daltons (average pore size ~20 nm or ~200 angstroms). The membrane was 85.6 cm long with 7 tubular channels for sludge flow. Regeneration was performed using a rinse-bleach-nitric acid-rinse method

at 60-80^{o}C requiring 5-hours to complete. Regeneration was only performed as necessary after the transmembrane pressure (TMP) became limiting to the required flow rate. No backwash procedure was used. Membrane is further characterized in Cicek (1998).

Measurement of MTBE and metabolite concentrations

Effluent MTBE and metabolites (tertiary butyl alcohol (TBA), tertiary butyl formate (TBF), methanol, methyl acetate, and acetone) were monitored daily using a heated purge and trap method employing a Tekmar Dohrmann (Cincinnati, OH) 3100 sample concentrator with a Hewlett Packard 6890 Series Plus GC and a DB-1 column (J&W Scientific, Folsom, CA).

Denaturing gradient gel electrophoresis (DGGE)

Mixed liquor from the MBR was sampled on a monthly basis during startup and initial steady-state conditions and characterized by DGGE. DNA was extracted using a Bio-101 (Vista, CA) FastDNA kit utilizing a bead-beating method as described in the manufacturer protocol. A 200 bp fragment of the V3 region of the 16S rDNA was polymerase chain reaction (PCR) amplified using the primers described in Muyzer *et al.* (1993) and the conditions described in Pruden *et al.* (in press).

DGGE was carried out with a D-Code 16/16 cm acrylamide gel system (BioRad, Hercules, CA) maintained at a constant temperature of 60°C in 6 L of 0.5x TAE buffer (20mM tris-acetate, 0.5mM EDTA, pH 8.0). Gradients were formed between 15% and 55% denaturant (100% denaturant defined as 7 M urea plus 40% v/v formamide) and the gels were run at 35 volts for 20 hours. Gels were stained in purified water containing ethidium bromide at 0.5 mg/L, and de-stained once in 0.5 x TAE. Images were documented using a GelDoc 2000 and Quantity One software (BioRad).

The central 1 mm^2 portions of the bands of interest were excised with razor blades and soaked in 36 µL purified water for 3 days at –20°C. One µL of this was used as template for PCR reaction, as described above. PCR products were purified using a GeneClean Spin Kit (Bio101). Sequencing was done by Davis Sequencing (Davis, CA) using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Foster City, CA).

RESULTS AND DISCUSSION

The MBR required a notably long start-up period of 120 days before stable effluent quality was obtained. Upon reaching steady-state, MTBE effluent concentrations were well below the lowest current drinking water standard of 5 µg/L. Tertiary butyl alcohol (TBA) the only MTBE intermediate that was observed during this study, was also below 5 µg/L. Table 1 summarizes performance of the system before and after start-up. Average MTBE effluent compared to the median MTBE effluent during start-up reveals a skew in the data set as well as a large standard deviation. This is indicative of the erratic initial performance with a general trend towards improvement as steady-state was attained.

Table 1. Summary of MBR effluent quality before and after start-up.

		Day –120 to 0 µg/L	Day 0-190 µg/L
MTBE	average	108	0.219
	standard deviation	247	0.188
	median	8.1	0.155
TBA	average	2.5	1.47
	standard deviation	2.8	1.16
	median	1.6	1.32

Figure 1 and Table 2 give a summary of the DGGE bands identified during start-up and steady-state of the reactor. The banding pattern remained identical throughout operation of the reactor with the exception of the earliest of the start-up samples. This sample was characterized by the absence of MBR band 3, and the presence of MBR band A. Identification of the bands by DNA sequencing revealed that at steady-state the culture was almost entirely dominated by a single group of organisms belonging to the *Sphingomonas* group of the α-Proteobacteria.

Dominance of the MBR culture by *Sphingomonas* was unexpected considering that the seed cultures consisted almost entirely of members of the *Cytophaga-Flexibacter-Bacterioides* (C-F-B) group of bacteria. Only one organism from the original seed culture was identified as belonging to the *Sphingomonas*. This organism was found in a porous-pot bioreactor which received MTBE and diisopropyl ether (DIPE) as primary substrates. Pruden et al. (in press) gives a complete phylogenetic overview of the seed cultures. Although the seed cultures were dominated by C-F-B organisms, none were found in the steady-state MBR culture. However, one organism belonging to the C-F-B group of bacteria was observed during start-up (MBR band A). Both the seed culture and the steady-state MBR culture contained one organism belonging to the *Nitrospina* division of bacteria (MBR band 3).

The stark contrast between the community composition of the seed culture compared with the MBR culture indicates that the membrane is exerting a selective pressure. Considering that the only C-F-B organism observed in this study was seen within the first sampling event indicates that the membrane has an immediate effect on the culture. Similarly, Cicek *et al.* (1999) observed a decline in the filamentous organisms resident to a membrane bioreactor when compared to an activated sludge reactor from the same seed culture. Loss of filamentous organisms was attributed to the high sheer stress exerted by the membrane. In a similar fashion, the seed culture consisting mostly of C-F-B organisms, originating from a quiescent flow regime, may not have been the best suited for membrane survival. This may explain the long start-up period that was required for this system to attain stable MTBE removal.

Figure 1. DGGE banding pattern observed consistently during MBR operation (a) and initially at start-up (b). Numbers or letters indicate band as identified in Table 2.

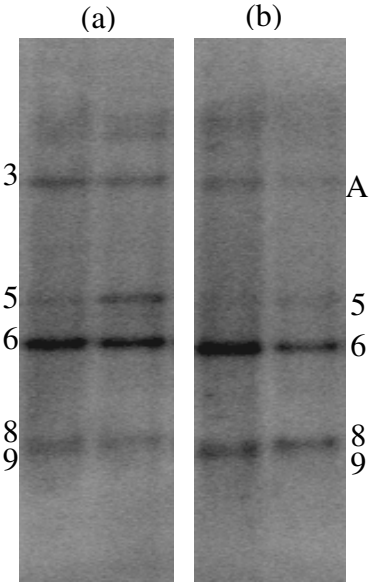

Table 2. Identification of DGGE bands. Highest match from the Ribosomal Database Project (RDP) is reported.

Band number	identity	RDP % similarity
3	*Nitrospina*	0.919
5	*Sphingomonas*	0.861
6	*Sphingomonas*	1.000
8	*Sphingomonas*	0.958
9	*Sphingomonas*	1.000
A	C-F-B	0.579

Although an overall change in culture composition was observed and maintained almost immediately, time was still required before the removal of MTBE stabilized. This is most likely because although selection by the membrane took place right away, adequate biomass levels had not yet been achieved. This makes sense since the dominant organisms in the steady-state MBR represented only a very small fraction of the seed culture. Volatile suspended solids (VSS) were measured below 100 mg/L for the first 90 days before finally starting to increase. By day 120, when steady-state removal of MTBE was attained, the VSS concentration had reached 1500 mg/L. After day 120, the VSS continued to increase steadily before finally leveling off in the range of 3000-3500 mg/L.

Sphingomonas are unique organisms, well-known for their exceptional capabilities in the degradation of xenobiotics, such as polycyclic aromatic hydrocarbons, chlorinated aromatics, dioxins, and pesticides, such as 2-4-D. *Sphingomonas* are aerobic gram negative rods belonging to the α–4 subclass of the α-Proteobacteria. They are characterized by the presence of glycosphingolipids in the outer membrane rather than the typical lipopolysaccharides present in nearly all other gram negative bacteria. The presence of glycosphingolipids in *Sphingomonas* are an evolutionary mystery considering that they are thought to be of eukaryotic origin (White *et al.*, 1996). The uncommon structure of their outer membranes renders them particularly hydrophobic, and lends to the increased bioavailability of several of the insoluble contaminants listed above. It is not unlikely that the exceptional structure of the *Sphingomonas* outer membrane has imparted an advantage to membrane survival in this study.

One MTBE degrading strain of *Sphingomonas* has been isolated in pure culture by Hanson *et al.* (1999), but was not subject to further characterization. More recently, *Sphingomonas* have been identified as a component of a mixed culture degrading MTBE and benzene, toluene, ethylbenzene, and p-xylene (Pruden *et al.*, submitted).

Sphingomonas have been observed to degrade other ethers as well, but by a di-oxygenase pathway (Schmidt *et al.*, 1992), rather than by the mono-oxygenase pathway that is currently understood. This may indicate that diverse pathways exist for MTBE degradation.

CONCLUSIONS

Sphingomonas, a group of gram negative bacteria possessing unique outer membrane characteristics were found to be dominant in a membrane bioreactor treating MTBE contaminated water. A striking difference between the MBR culture and the original seed culture indicated that the membrane exerted a selective pressure on the microorganisms present in the seed. While a long start-up period may be attributed to the shift in culture composition, the steady-state MBR effluent quality was excellent, with MTBE concentrations well below the most stringent drinking water standards.

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