

Assessing the correlation between anaerobic toluene degradation activity and *bssA* concentrations in hydrocarbon-contaminated aquifer material

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Abstract The assessment of biodegradation activity in contaminated aquifers is critical to demonstrate the performance of bioremediation and natural attenuation and to parameterize models of contaminant plume dynamics. Real time quantitative PCR (qPCR) was used to target the catabolic *bssA* gene (coding for benzylsuccinate synthase) and a 16S rDNA phylogenetic gene (for total *Bacteria*) as potential biomarkers to infer on anaerobic toluene degradation rates. A significant correlation ($P = 0.0003$) was found over a wide range of initial toluene concentrations (1–100 mg/l) between toluene degradation rates and *bssA* concentrations in anaerobic microcosms prepared with aquifer material from a hydrocarbon contaminated site. In contrast, the correlation between toluene degradation activity and total *Bacteria* concentrations was not significant ($P = 0.1125$). This suggests that qPCR targeting of functional genes might offer a simple approach to estimate in situ biodegradation activity, which would enhance site investigation and

modeling of natural attenuation at hydrocarbon-contaminated sites.

Keywords Benzylsuccinate synthase · Natural attenuation assessment · Anaerobic · Toluene

Introduction

Bioremediation and Monitored Natural Attenuation (MNA) can be practical and cost-effective approaches to manage groundwater contamination by petroleum product releases (Alvarez and Illman 2006). However, microorganisms with the required catabolic capacity (and ability to express it under site-specific conditions) may not be present in sufficient numbers, which renders these environmental biotechnologies selectively applicable and occasionally marginally effective. Consequently, extensive site characterization and documentation of natural biodegradation processes are necessary to gain regulatory approval and public endorsement for the use of MNA as a remediation approach.

Insight on in situ biodegradation activity can be provided by MNA forensic approaches such as geochemical footprints, numerical modeling and compound specific isotope analysis (CSIP). However, these approaches can be labor-intensive and are based on indirect evidence of contaminant degradation that do not always yield unequivocal evidence. For

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instance, background substrates and redox processes that consume electron acceptors can confound geochemical footprints; numerical modeling may be challenged by incomplete understanding of site heterogeneities; and CSIP has potential limitations regarding its sensitivity, resulting in a lack of stable isotope fractionation despite biodegradation taking place (Illman and Alvarez 2009). Thus, these forensic techniques are not universally applicable, and there is a need for additional in situ biodegradation assessment approaches.

MNA performance assessment can be complemented by molecular genetic approaches to assess indigenous microorganisms and their catabolic genes involved in the degradation of target pollutants (Allen et al. 2007). Real time quantitative polymerase chain reaction (qPCR) enables detection and quantification of both structural and functional genes associated with contaminant degradation. This can be utilized to demonstrate MNA by establishing the presence and increased concentration of specific degraders in the active zone compared to background samples (Scow and Hicks 2005; Weiss and Cozzarelli 2008). For example, higher concentrations of reductive dehalogenase genes *tceA*, *vcrA*, and *bvcA* (associated with *Dehalococcoides* spp.) in enrichment cultures and aquifers exhibiting reductive dechlorination of trichloroethylene provided evidence of the critical role of this genus in the bioremediation process (Krajmalnik-Brown et al. 2004; Ritalahti et al. 2006).

Various correlations between contaminant removal and associated catabolic gene expression have been reported. For example, a correlation between mercury volatilization rates and mercuric reductase (*merA*) transcript levels was observed in environmental samples (Nazaret et al. 1994; Jeffrey et al. 1996). Also, anaerobic respiration rates (assessed per fumarate reduction or Fe(III)) were positively correlated with the expression of *omcB* (an outer membrane *c*-type cytochrome gene involved in Fe(III) reduction) and *frdA* (coding for fumarate reductase) in *Geobacter sulfurreducens* (Chin et al. 2004). Naphthalene degradation rates under aerobic conditions have also been reported to correlate positively with the expression of *nahA* (coding for naphthalene dioxygenase) (Fleming et al. 1993; Park and Crowley 2006). Nevertheless, the usefulness of specific biomarkers to assess anaerobic biodegradation of aromatic hydrocarbons has received limited attention.

A qPCR method was developed by Beller et al. (2002) to detect and quantify the catabolic gene *bssA*, which codes for benzylsuccinate synthase—a key enzyme that initiates anaerobic toluene degradation by attaching fumarate to the methyl group of toluene to form benzyl succinate (Biegert et al. 1996). This gene has been detected in toluene degrading cultures under a wide variety of anaerobic electron accepting conditions, including denitrifying (Biegert et al. 1996; Beller and Spormann 1997), sulfate-reducing (Beller and Spormann 1997; Rabus and Heider 1998), anoxygenic phototrophic (Zengler et al. 1999), iron-reducing (Kane et al. 2002) and methanogenic conditions (Beller and Edwards 2000; Da Silva and Alvarez 2004). Recently, Winderl et al. (2007) designed a qPCR assay based on additional *bssA* sequences to target a more diverse community of known and unknown anaerobic toluene degraders. Thus, *bssA* may be an important catabolic biomarker gene for assessing anaerobic toluene and xylenes degradation potential (Beller et al. 2008; Winderl et al. 2007, 2008). This biomarker has been used to quantify the vertical distribution of anaerobic toluene degraders in a sulfidogenic aquifer contaminated with monoaromatic hydrocarbons, and to show that higher *bssA* concentrations occurred within the contaminated zone (Winderl et al. 2008). However, the usefulness of *bssA* to directly estimate alkylbenzene degradation rates in environmental samples has not yet been explored.

In this paper, we demonstrate a direct correlation between *bssA* concentrations and anaerobic toluene degradation rates in anaerobic microcosms prepared with hydrocarbon contaminated aquifer material. Our data suggests that functional biomarker targeted qPCR can be used to estimate in situ degradation rates, in turn providing valuable support for MNA selection decisions and performance assessment.

Materials and methods

Sample collection and analysis

Samples were collected from a hydrocarbon-contaminated site located at the Mission Valley Terminal Site, San Diego, California. Aquifer material was collected from the top of the saturated zone underlying an active manifold and fuel tank area that has been exposed to multiple releases of gasoline and

diesel of varying volumes and hydrocarbon compositions over decades. The aquifer sample was collected when drilling the well using a Hollow Stem Auger from 20.5 to 23.8 feet below ground surface. Water was collected from within the auger after soil sampling and the core was immediately shipped to Rice University and upon receipt the core was stored at 4°C. Groundwater and sediment chemical analyses were performed by TestAmerica Laboratories, Inc., in Irvine CA and Houston, TX (Table 1).

The aquifer material was a dark grayish brown, moist, fine to medium grained clayey sand with neutral pH and low organic carbon content (<0.2%). The extractable fuel hydrocarbons (C13–C40) concentration was 66 mg/kg, whereas the concentration of volatile fuel hydrocarbons GRO (C4–C12) was 38 mg/kg. Benzene, toluene, ethylbenzene, xylenes and methyl-*tert*-butyl ether (MTBE) were present at a concentration of 100, 240, 430, 1200 and 360 µg/kg, respectively. Other volatile organics such as diisopropyl ether (DIPE), ethyl *tert*-butyl ether (ETBE), *tert*-amyl methyl ether (TAME) or *tert*-butyl alcohol (TBA) were not detected in the aquifer sample.

Table 1 Local characteristics of aquifer from where samples were collected for this study

Constituent	Concentration	
	Aquifer material (mg/kg)	Groundwater (mg/l)
EFH (C13–C40)	66	NA
GRO (C4–C12)	38	NA
TPH purgeable	NA	6.1
Benzene	0.10	1.1
Toluene	0.24	0.28
Ethylbenzene	0.43	0.34
Total xylenes	1.20	0.48
MTBE	0.36	0.24
Methane	ND	0.23
TOC	BRL	4.9
Iron	5680	0.85
Nitrate	ND	<0.25
Sulfate	36.1	110
DO	NA	0.1
ORP (mV)	NA	–13.0
Soil pH	7.38	NA

NA not analyzed, ND not detected, BRL below reporting limit (0.2%)

Sulfate was measured at 36.1 mg/kg, whereas total iron content was as high as 5,680 mg/kg and nitrate was not detected. Groundwater analysis was also conducted. Benzene, toluene, ethylbenzene, xylenes and MTBE were present. Dissolved oxygen and oxidation reduction potential (ORP) values, as well as the presence of methane (0.23 mg/l or 14 µM) indicated strongly anaerobic conditions. Although the prevalent electron accepting process could not be discerned, iron-reducing conditions appear to be important at this site (Table 1).

Microcosm preparation and determination of biodegradation rates

Anaerobic microcosms were prepared with 20 g of aquifer sediment and 80 ml of bicarbonate-buffered medium in 240 ml amber glass bottles capped with Mininert valves (Sigma–Aldrich, St. Louis MO). The composition of the medium was similar to the synthetic groundwater described by von Gunten and Zobrist (1993) with some modifications. As described by Beller et al. (2002), lactate was excluded and the bicarbonate, nitrate, ammonium, and phosphate concentrations were 7.3, 5.3, 0.3 and 0.02 mM, respectively. Before use, the medium was deoxygenated and equilibrated with an anaerobic chamber atmosphere (N₂ = 90%; CO₂ = 5%; H₂ = 5%) for 3 days. Microcosms were prepared in triplicate inside an anaerobic chamber (COY laboratories, Grass Lake, MI) and amended with toluene concentrations of 1, 5, 10, 25, 50, or 100 mg/l. To account for volatilization losses, abiotic controls were also prepared with autoclaved sediment and poisoned with a commercial biocide (Kathon CG/ICP) at 10 ml/l. Microcosms were incubated in the dark at room temperature (~22°C). Toluene concentrations were determined by headspace analysis (50 µl) with a Hewlett-Packard 5890 series II gas chromatograph equipped with a Supelco 60/80 Carboxpack column and a flame ionization detector.

Toluene biodegradation rates were determined by linear regression of the linear region of concentration versus time data, which typically occurred after a 1–3-day lag period, as described by Hutchins (1997):

$$C_0 - C = kt$$

Where C_0 is the initial toluene concentration at the beginning of the selected linear-decay period, C is the final concentration at the end of selected time period,

k is the zero-order degradation rate, and t is the time interval. Entire microcosms were sacrificed for qPCR analysis immediately after complete toluene removal.

To monitor changes in gene copy numbers during the incubation period, separate microcosms were prepared similarly with a constant initial toluene concentration of 5 mg/l; these microcosms were also sacrificed for qPCR analysis after selected time intervals.

DNA extraction

Microcosms were centrifuged and the pelleted aquifer material was used for DNA extraction using the MoBio Power Soil™ kit (Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and purity of DNA was measured using a spectrophotometer at 260 nm (Amersham Biosciences, Model Ultraspec 2100 Pro, Piscataway, NJ, USA). Bacteriophage λ DNA (500 bp, Sigma–Aldrich, St. Louis, MO, USA) was added (20 ng) as an internal standard to the sediment samples prior to DNA extraction, to determine DNA recovery efficiency (Beller et al. 2002). Extracted DNA was stored at -20°C prior to qPCR analysis.

Real-time quantitative PCR

qPCR was used to quantify the concentration of *bssA* and total *Bacteria* 16S rDNA. Each qPCR reaction mixture contained 1 \times Taqman PCR Master Mix

(Applied Biosystems, Foster City, CA, USA), 0.625 μM of forward and reverse primers, 0.25 μM of probe, 4 μl of sample DNA and sterile water to make a final volume of 20 μl . The primers and probes (Table 2) used in this study were described by Beller et al. (2002) and obtained from Integrated DNA Technologies (Coralville, IA, USA). qPCR was performed using a Sequence Detector (Model ABI 7500, Applied Biosystems) with the following temperature conditions: 50°C for 2 min, followed by 95°C for 10 min and 40 cycles at 95°C for 15 s, and 60°C for 1 min. Calibration curves were prepared using (10^1 – 10^8 gene copies/ μl) genomic DNA of *Thauera aromatica* (ATCC# 700265D) and of *Escherichia coli* (ATCC# 10798D-5) for the quantification of *bssA* and *Bacteria* 16S rRNA gene, respectively. Calibration curves yielded r^2 values of 0.997 or greater.

The biomarker concentrations in each sample were estimated using the following equation described by Da Silva and Alvarez (2008):

$$\text{Gene copy}/\mu\text{l} = [(\mu\text{g DNA}/\mu\text{l}) / (5.3 \times 10^6 \text{ bp/genome})] \times (9.1257 \times 10^{14} \text{ bp}/\mu\text{g DNA}) \times (\text{genes/genome}).$$

We assumed a genome size of 5.3×10^6 base pairs (bp), 9.1257×10^{14} bp/ μg DNA (i.e., $(6.022 \times 10^{17} \text{ Da}/\mu\text{g of DNA}) / (660 \text{ Da}/\text{bp})$) and 7 gene copies per genome for *E. coli* (<http://www.genomesonline.org>). Similarly, to calculate the *bssA* gene copy number, a genome size of 4.6×10^6 bps and one copy of *bssA* per genome was used for *T. aromatica*. The qPCR analysis of the internal standard λ was performed to

Table 2 Primers and probe sequences used for real time quantitative PCR

Target	Primers and probe ^a	Sequences (5'–3')
<i>Bacteria</i> ^b (16S rRNA)	Forward	CGGTGAATACGTTTCYCGG
	Reverse	GGWTACCTTGTTACGACTT
	Probe	FAM-CTTGTACACACCGCCCGTC-BHQ1
<i>bssA</i> ^c	Forward	ACGACGGYGGCATTTC
	Reverse	GCATGATSGGYACCGACA
	Probe	FAM-CTTCTGGTCTTCTGCACCTTGGACACC-TAMRA
Bacteriophage λ ^d	Forward	ACGCCACGCGGGATG
	Reverse	AGAGACACGAAACGCCGTTT
	Probe	TET-ACCTGTGGCATTGTGTCTGCCG-TAMRA

^a The reporter dye used were FAM (6-carboxyfluorescein) or TET (tetrachloro-6-carboxyfluorescein) and the quencher dye was either TAMRA (6-carboxy tetramethyl rhodamine) or BlackHole Quencher-1

^b *Bacteria* primers and probe were designed by Suzuki et al. (2000)

^{c, d} Primers and probes were designed by Beller et al. (2002)

correct the values of the *bssA* and 16S rRNA gene copy numbers. DNA recovery ranged from 6.1 to 115%. Such variations in DNA recoveries from soil are common (Zhou et al. 1996) and often reflect the binding of humic acids and other sample impurities that interfere with *Taq* polymerase during PCR analysis (Porteous et al. 1997). Recoveries lower than 100% were normalized to the fraction recovered and no correction was made for the samples in which λ DNA recovery was more than 100%. Minimum detection limits were 2 gene copies/ μ l for both total *Bacteria* and *bssA*. Consumption of about 0.5 mg/l toluene was required to obtain a quantifiable signal.

Results and discussion

Toluene degradation activity and correlation with biomarker concentrations

Toluene degradation was observed in anaerobic microcosms over a wide range of initial toluene concentrations (1–100 mg/l) but not within the sterile control (Fig. 1). Toluene was rapidly depleted (generally within 7 days of incubation), which suggests that the anaerobic community from this contaminated site was acclimated in situ to toluene. Given the

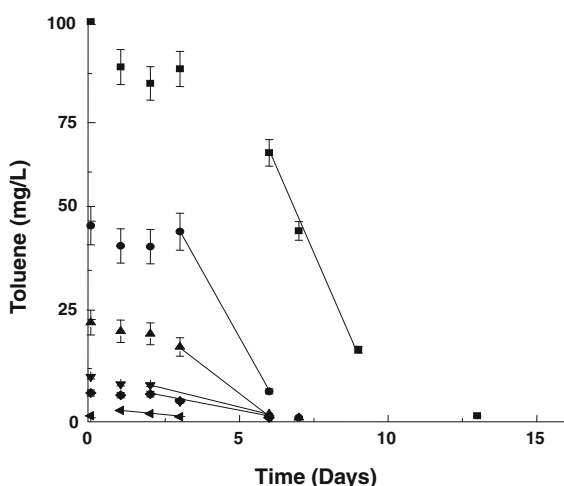


Fig. 1 Toluene degradation in anaerobic microcosms with different initial concentrations: (◄) 1 mg/l, (◆) 5 mg/l, (▼) 10 mg/l, (▲) 25 mg/l, (●) 50 mg/l, and (■) 100 mg/l. Error bars represent \pm one standard deviation from the mean of triplicate microcosms. Losses from sterile controls were less than 5% (not shown)

objective of this study, to explore correlations between biodegradation rates with biomarker concentrations, anaerobic electron acceptors (or their reduced products) were not monitored to conduct electron balances and characterize better the electron accepting conditions. However, methane accumulated in replicate microcosms at levels up to 7.4 mmol, indicating strongly anaerobic conditions.

A significant linear relationship ($r^2 = 0.99$; $P = 0.0011$) was observed between the amount of toluene degraded and the resulting *bssA* gene copy numbers (Fig. 2a). Thus, bacteria harboring *bssA* grew in proportion to the amount of toluene consumed, with a cell yield coefficient of approximately 5×10^9 cells/mg toluene (i.e., from the slope of Fig. 2a: 2×10^7 cells g-soil $^{-1}$ /mg-toluene l $^{-1}$ \times 20 g-soil/0.08 l per microcosm). The corresponding linear correlation with *Bacteria* 16S rRNA gene was not significant ($P = 0.148$) (Fig. 2b), indicating that the 16S rDNA biomarker was less sensitive to track the proliferation of anaerobic toluene degraders.

The concentrations of *bssA* and *Bacteria* 16S rDNA biomarkers were monitored during and after toluene degradation in microcosms that had been prepared for stability assessment. Copy numbers of *bssA* increased several folds (more than 3.5 log) as toluene degraded, and decreased after toluene was depleted (Fig. 3). The observed increases in *bssA* copies are consistent with reported cell yields and generation times for toluene-degrading, denitrifying bacteria (Beller et al. 2002). For example, cell yields for toluene-degrading, denitrifying bacteria have been reported to be on the order of 100 g cells/mol toluene (Spormann and Widdel 2000). Assuming that the mass of one cell is about 1 pg (Neidhardt 1987) and considering the mass of toluene consumed (6 μ mol), an increase up to the order of 10^8 – 10^9 cells is expected. Assuming an average of one copy of *bssA* per cell, the observed 100- to 1,000-fold increase in 3 days is reasonable; reported doubling times of denitrifying bacteria growing on toluene (Spormann and Widdel 2000) suggest that a 1,000-fold increase would occur in around 2.5 days.

These results corroborate the sensitivity of the *bssA* biomarker to infer on anaerobic toluene degradation activity. *Bacteria* 16S rRNA gene was a much less sensitive biomarker for this purpose. Other functional genes have been reported to be sensitive biomarkers in response to biodegradation of priority

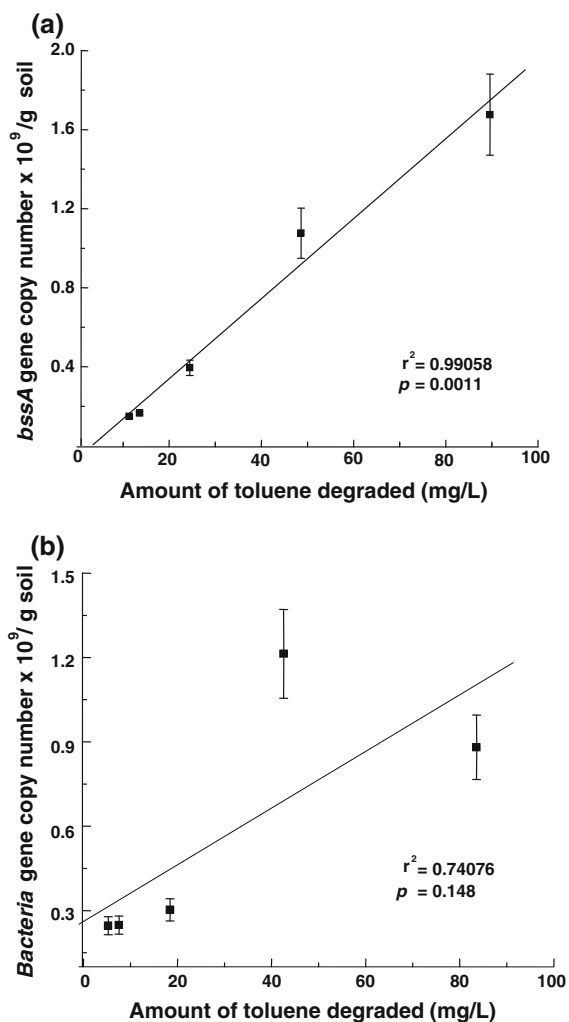


Fig. 2 Final biomarker concentrations for **a** *bssA* and **b** *Bacteria* 16S rRNA increased with amount of toluene degraded. Error bars represent \pm one standard deviation from the mean of triplicate microcosms

pollutants, including *phe* (coding for phenol hydroxylase that degrades various monoaromatic hydrocarbons) (Nakatsu et al. 2005) and *tfdA* (coding for the Tfd A class III enzyme that degrades 4-chloro-2-methylphenoxyacetic) (Baelum et al. 2006). However, total *Bacteria* biomarkers have been reported to correlate better than *Dehalococcoides* sp. functional and phylogenetic biomarkers with trichloroethylene dechlorination rates (Da Silva and Alvarez 2008), which forewarns that some functional biomarkers may be miss-targeted or under-expressed, and might not always be reliable indicators of biodegradation activity.

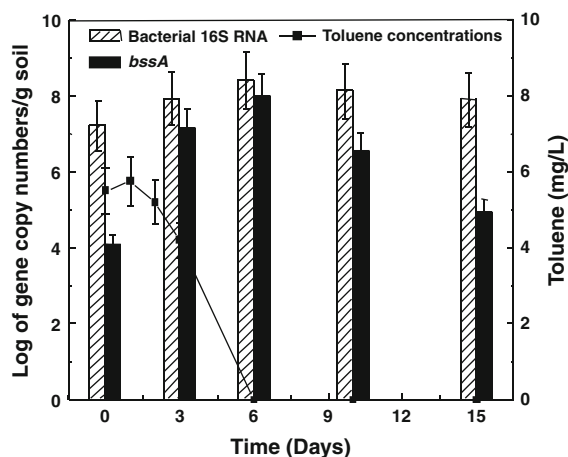


Fig. 3 Changes in target gene copy numbers (bars, left axis) during anaerobic toluene degradation (line, right axis). Error bars represent \pm one standard deviation from the mean of triplicate microcosms

Consistent with results presented in Figs. 2 and 3, a significant correlation ($r^2 = 0.996$; $P = 0.0003$) was found between the concentration of *bssA* and the anaerobic toluene degradation rates (Fig. 4a), whereas the corresponding correlation with the *Bacteria* 16S rRNA biomarker was not significant ($r^2 = 0.789$; $P = 0.1125$) (Fig. 4b). A tighter correlation to degradation activity might be obtained with *bssA* transcripts. However, quantifying transcripts is a difficult task due to the relative instability of mRNA and the lack of standardized isolation methods. In light of the high correlation obtained using gene copy numbers, we felt it was unnecessary to quantify *bssA* transcripts.

We recognize that the presence of specific degraders and the pertinent catabolic genes does not guarantee their expression, and that there may be numerous site-specific factors that could confound the correlation between biodegradation rates and biomarkers concentrations (e.g., mass transfer limitations, nutrients availability, suboptimal pH or temperature, and presence of inhibitory compounds). However, many confounding factors are likely to affect similarly both degradation rates and biomarker enrichment (through microbial growth or decay) over the large temporal scales that are relevant to bioremediation and MNA. For example, if nutrients and growth factors are limiting, both the concentration of specific degraders and the degradation rates will eventually decrease, possibly following a correlated pattern. Overall, our data suggest that the *bssA* biomarker might be useful

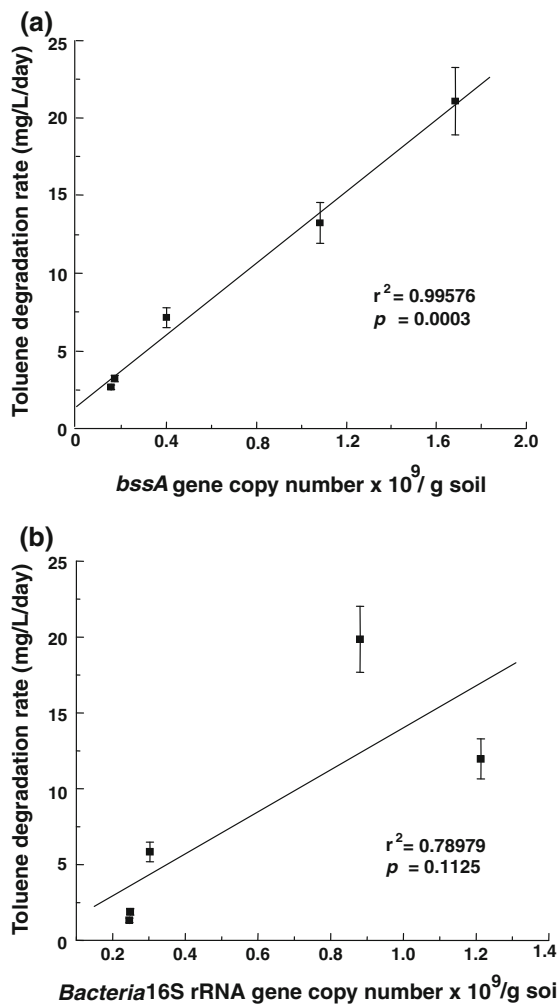


Fig. 4 Correlations of anaerobic toluene biodegradation rate versus **a** *bssA* gene copy number and **b** *Bacteria* 16S rRNA gene copy number. Error bars represent \pm one standard deviation from the mean of triplicate microcosms

not only to infer anaerobic toluene biodegradation potential, but also to estimate in situ biodegradation rates and assess their spatial variability in fuel contaminated subsurface environments.

Conclusion

There is a growing need for improved performance assessment and demonstration of anaerobic biodegradation for hydrocarbon-impacted aquifers being treated by or considered for monitored natural attenuation and bioremediation. One promising

approach is the use of molecular methods to establish correlations between in situ biodegradation activity and specific gene biomarker concentrations. In this work, a qPCR-based biomarker targeting the *bssA* catabolic gene (coding for benzyl succinate synthase) was shown to be a sensitive indicator to assess the proliferation of anaerobic toluene degraders and infer on anaerobic toluene degradation rates. Overall, these results suggest that functional biomarker gene targeted qPCR might offer a relatively simple and inexpensive approach to estimate in situ toluene degradation activity and characterize its spatial variability to enhance site investigation and assessment of natural attenuation at hydrocarbon-contaminated sites. However, further work is required to investigate how site-specific hydrogeologic and geochemical factors may affect the accuracy of such inferences and delineate better the merits and limitations of functional and phylogenetic gene biomarkers for forensic analysis of bioremediation and MNA.

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