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# Application of the 5′ Fluorogenic Exonuclease Assay (TaqMan) for Quantitative Ribosomal DNA and rRNA Analysis in Sediments

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**In this study, we report on the development of quantitative PCR and reverse transcriptase PCR assays for the 16S rRNA of *Geobacter* spp. and identify key issues related to fluorogenic reporter systems for nucleic acid analyses of sediments. The lower detection limit of each assay was 5 to 50 fg of genomic DNA or ≤2 pg of 16S rRNA. TaqMan PCR spectral traces from uncontaminated, amended aquifer sediments were significantly lower ($P < 0.0002$) than traces for the external standard curve. We also observed a similar, significant decrease in mean quencher emissions for undiluted extracts relative to those for diluted extracts ($P < 0.0001$). If PCR enumerations were based solely upon the undiluted sample eluant, the TaqMan assay generated an inaccurate result even though the threshold cycle ($C_t$) measurements were precise and reproducible in the sediment extracts. Assay accuracy was significantly improved by employing a system of replicate dilutions and replicate analyses for both DNA and rRNA quantitation. Our results clearly demonstrate that fluorescence quenching and autofluorescence can significantly affect TaqMan PCR enumeration accuracy, with subsequent implications for the design and implementation of TaqMan PCR to sediments and related environmental samples.**

Nucleic acid technology has initiated a new era in environmental microbiology by providing specific, sensitive detection of (uncultured or unculturable) microorganisms in chemically and biologically complex backgrounds. The ultimate application of nucleic acid technology is to provide knowledge of the absolute composition, abundance, and structure of microbial communities and the dynamics of individual populations, organisms, or genes within that community. The dominant technique in environmental molecular microbiology is PCR, and at the forefront of PCR methods are quantitative PCR (qPCR) and reverse transcriptase PCR (RT-PCR) (12, 19–21, 25, 31, 37, 47).

Typical qPCR techniques utilize approaches originally developed in clinical laboratory settings (e.g., see references 3, 10, 18, 24, and 38). In this vein, the 5′ fluorogenic exonuclease (i.e., TaqMan) assay represents the latest development in real-time qPCR methods (2, 11, 16, 17, 26, 30, 32, 34–36) and instrumentation (5, 22, 33, 44). By utilizing an internal probe in addition to standard PCR amplification primers, TaqMan chemistry combines the amplification power of PCR with the specificity and verification of Southern hybridization. Labeling the internal probe with fluorescent dyes provides in-tube, real-time detection of PCR product accumulation during each amplification cycle and at very early stages in the amplification process.

Environmental samples have levels of chemical and genetic complexity not normally encountered in tissue and/or physiological samples or pure cultures, which may affect the ability of TaqMan PCR to quantify RNA and DNA in these matrices. Several recent reports have suggested that TaqMan chemistry can be successfully applied to DNA analysis in concentrated water samples (4, 42, 43). A routine terrestrial or benthic sample, however, contains organic contaminants, metals, chelators, humic acids, or other inhibitory compounds that can copurify with nucleic acids and complicate the amplification process (46, 48). These inhibitors may also interfere with fluorescence detection, independent of their effects on *Taq* polymerase or RT. In this study, we report on the development of quantitative PCR and RT-PCR assays for the 16S rRNA of *Geobacter* spp., identify key issues related to fluorogenic reporter systems for nucleic acid analyses in sediments, and offer some practical solutions to measure and account for potential obfuscation of TaqMan PCR data under these circumstances.

## MATERIALS AND METHODS

**Bacterial cultures.** *Geobacter chapellei* and an undescribed environmental *Geobacter* species (tentatively designated "*Geobacter bemidjiensis*") obtained from uncontaminated Bemidji, Minn., aquifer sediment (39) were used as reference and calibration standards for all experiments. *G. chapellei* was cultivated as described in reference 7, and "*G. bemidjiensis*" as described in reference 39.

**Sediments.** Anaerobic, uncontaminated sediment cores from a Bemidji, Minn., aquifer (39) were collected in 1997 with a drill rig or hand auger and transported immediately to the laboratory. Sediment cores were homogenized and transferred to storage bottles in an $N_2$-filled glove bag. Forty-gram (dry weight) subsamples were placed in serum bottles (60 ml) under $N_2$, sealed with thick butyl rubber stoppers, and removed from the glove bag, and the headspace was flushed with $N_2$-$CO_2$ (93:7). Additional sediments were obtained from 182- to 190-m depths at Cerro Negro, N.Mex., as described elsewhere (13).

**Sediment microcosms.** Sediment amendments were designed to test various electron shuttle and Fe(III) reduction hypotheses (27), which will be reported elsewhere. Uncontaminated Bemidji aquifer sediments (40 g [dry weight]) were amended with 5 mM (final concentration in 40 g) formate. Sediment microcosms were prepared in triplicate and incubated at 20°C for 82 days. Subsamples from each microcosm were aseptically and anaerobically taken in an $N_2$-filled glove bag for molecular analyses at 49 days. Sediment that was not amended with an

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TABLE 1. Primers and probes used for the development of *Geobacter* TaqMan PCR assays of sediments

| Primer or probe | Sequence | $T_m$[a] (°C) | Position (*G. chapellei* numbering) |
|---|---|---|---|
| Primers | | | |
| 361F | 5′-AAGCCTGACGCASCAA-3′ | 56 | 361–376 |
| S-δ401F-20 | 5′-AASCCTGACGCAGCRACGCC-3′ | 71 | 361–380 |
| 561F | 5′-GCGTGTAGGCGGTTTCTTAA-3′ | 57 | 557–576 |
| 685R | 5′-ATCTACGGATTTCACTCCTACA-3′ | 52 | 664–685 |
| S-δ683aR-20 | 5′-TCTACGGATTTCACTCCTACAC-3′ | 52 | 663–684 |
| 825R | 5′-TACCCGCRACACCTAGTTCT-3′ | 54 | 802–821 |
| 1392R | 5′-ACGGGCGGTGTGTRC-biotin-3′ | 57 | 1374–1388 |
| Probes | | | |
| Gbc1 | 5′-FAM-CACTTCCTGGGTTGAGCCCAG-TAMRA-3′ | 63 | 593–613 |
| Gbc2 | 5′-FAM-CTCAACCCAGGAAGTGCATTGGATAC-TAMRA-3′ | 65 | 598–623 |
| Eub1 | 5′-FAM-GTATTACCGCGGNTGCTGGC-TAMRA-3′ | 66 | 497–516 |

[a] $T_m$ as calculated with Perkin-Elmer Primer Express 1.0 software.

electron donor served as the background and/or negative control sample for TaqMan RNA quantitation (see below).

**Amended sediments.** Fine-grained pure quartz sand and Bemidji aquifer sediment were sterilized by autoclaving for 1 h and then exposed to 260-nm UV light for 1 h. Sterilized sediments were seeded with known densities of *Geobacter* cells as determined by acridine orange direct counting. Samples were prepared in triplicate, with cell counts ranging from $2.9 \times 10^7$ to <1 cell per g.

**DNA and RNA standards.** *G. chapellei* cells were collected by centrifugation and genomic DNA was isolated by a standard hexadecyltrimethylammonium bromide procedure (1). Genomic DNA was sheared to 4 to 10 kbp in size by ballistic disintegration for 1 min at 5,000 oscillations $s^{-1}$ in an eight-place bead beater (BioSpec Products, Inc., Bartlesville, Okla.). After the DNA was sheared, DNA concentrations were determined by fluorometry and sizes were determined with 1.2% agarose (SeaKem GTG, FMC, Rockland, Maine) gels in 1× Tris-acetate-EDTA running buffer, both containing ethidium bromide. "*G. bemidjiensis*" DNA was isolated with a MoBio Soil DNA extraction kit (which includes a bead-beater lysis step) according to the manufacturer's instructions (MoBio Laboratories, Inc., Solana Beach, Calif.).

Total RNA and 16S rRNA were isolated from *Geobacter* cells by a guanidium isothiocyanate:phenol:sarkosyl method as described elsewhere (8). 16S rRNA was selectively recovered from total RNA extracts utilizing a PolyA Tract mRNA purification system (Promega Corp., Madison, Wis.) and universal 16S oligonucleotide 1392R (Table 1). After 16S rRNA capture, samples were treated with amplification-grade DNase I as specified by the manufacturer (Life Technologies, Gaithersburg, Md.), and the DNase was removed by phenol-chloroform extraction. Purified RNA was then ethanol precipitated, resuspended in diethyl pyrocarbonate-treated water, quantified by UV absorbance, and stored at −80°C.

**DNA and RNA isolation from sediments.** Total genomic DNA from seeded and unseeded sand and aquifer sediments was extracted with a FastDNA Spin kit for soil (BIO 101, La Jolla, Calif.) according to the manufacturer's instructions. During the development of individual TaqMan assays and for the analysis of Cerro Negro sediments, single 0.5-g aliquots of sediment were processed and eluted in 50 μl of sterile water and two dilution series (undiluted, 1:100, and 1:500) prepared from the single extract (six TaqMan data points). For seeded sediments and RNA quantitation in Bemidji mesocosms, two independent extractions were performed and two independent dilution series from each extract were generated (12 data points). Template DNA or RNA was then assayed by TaqMan or limiting-dilution PCR as described below.

Total RNA was isolated from Bemidji sediments with a modified FastDNA (BIO 101) protocol. Briefly, 0.5 g of sediment aliquots was lysed by ballistic disintegration and precipitated with protein precipitation solution according to the manufacturer's directions. After protein removal, the supernatant was directly precipitated with 2 volumes of ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. 16S rRNA was recovered by affinity purification as described above and stored at −80°C.

**Reverse transcription of 16S rRNA.** 16S rRNA was serial diluted in a 5- or 10-fold series immediately prior to reverse transcription, such that the first sample in the series represented 5% of the purified 16S rRNA eluant. All RT and PCR analyses were performed in duplicate. Ten microliters of 16S rRNA (concentrated or diluted), 2 pmol of reverse primer, and 1.5 μg of T4 gene 32 protein (Boehringer Mannheim) were heat denatured in 12 μl (total volume) at 70°C for 10 min. The reverse primer used for cDNA synthesis was the same reverse primer that was used for cDNA amplification by PCR and depended upon the specific TaqMan or PCR assay being tested (below). After heat denaturation, reverse transcription reaction mixtures were assembled in a 20.5-μl total volume, which included 0.5 μl of RNase Inhibitor (Life Technologies) and 1 μl of Moloney murine leukemia virus RT (Life Technologies). RT reaction mixtures were incubated for 50 min at 42°C and then heat inactivated at 100°C for 5 min. Two microliters from each reverse transcription reaction mixture was then used as a template for quantitative PCR (below).

RNase-treated controls were always performed to confirm RNA amplification and detection. Ten-microliter aliquots of concentrated 16S rRNA were treated with 10 μg of RNase A (10 mg $ml^{-1}$; Sigma, St. Louis, Mo.) for 15 min at 37°C before the RT assays were initiated. Tenfold serial dilutions of *G. chapellei* 16S rRNA served as a positive control and calibration curve for quantitative RT-PCR analyses.

**Limiting-dilution PCR.** The salient feature of limiting-dilution PCR is that we make no assumptions of amplification efficiency (as with competitive or most-probable-number [MPN]-PCR methods). Briefly, we acknowledge that all enumerations are relative to an (external, idealized) standard, such that every enumeration is only an estimate; the PCR assay has a known lower detection limit, but not necessarily single-copy sensitivity; we use and prefer the dilution-to-extinction concept but do not use MPN statistics; we make extensive use of amended controls to estimate the extent of PCR inhibition and minimum detection limits in the environmental sample; we make extensive use of external standards to calibrate the enumeration and estimate the extent of PCR inhibition; and we perform replicate nucleic acid extractions from the sample, with replicate serial dilutions prepared from each nucleic acid extract prior to the PCR. The basic experimental design for each unknown sample consists of two nucleic acid extractions, with two dilution series from each extract, and with a single PCR performed at each dilution point. A more detailed discussion of limiting-dilution PCR is found in reference 6.

PCR primers S-δ401F-20 and S-δ683aR-20 (Table 1) were synthesized by Keystone Laboratories (Camarillo, Calif.). PCR amplification was carried out with a 25-μl total volume, utilizing an MJ Research (Watertown, Mass.) Tetrad Thermal cycler and 0.2-ml thin-walled reaction tubes. The final reaction conditions were 2 μl of cDNA, 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 0.2 μM forward and reverse primers, and 0.625 U of *Taq* polymerase (Perkin-Elmer, Foster City, Calif.) which had been pretreated with TaqStart antibody at the recommended concentration (Sigma, St. Louis, Mo.). Assembled reaction mixtures were heated to 80°C for 5 min (hot start) and amplified with 5 cycles at 94°C for 40s, 60°C for 10 s, and 72°C for 75 s, followed by 40 cycles at 94°C for 12 s, 65°C for 10 s, and 72°C for 80 s with a 2-s extension per cycle. A final 20-min, 72°C extension was performed before the reaction mixtures were chilled to 4°C. The entire contents of each PCR mixture were analyzed on 1% NuSieve–1% Seakem GTG agarose (FMC Bioproducts, Rockland, Maine) gels in 1× Tris-acetate-EDTA running buffer, both containing ethidium bromide, and gel images were captured with a Bio-Rad (Hercules, Calif.) Fluor-S imager and Molecular Analyst software. The external standard curve was established with 500 pg of *G. chapellei* 16S ribosomal DNA

(rDNA) as template (in 2 μl), utilizing an appropriate dilution series of positive control template (to 5 fg of target).

**TaqMan primer and probe design.** TaqMan PCR utilizes fundamentally different chemical and thermal cycling conditions than standard PCR. The inclusion of an internal fluorescence resonance energy transfer (FRET) probe likewise constrains the design of PCR primers. Therefore, the PCR primers and reaction conditions for quantitative TaqMan PCR are slightly different than qPCR conditions employed for the limiting-dilution technique. We developed two sets of PCR primers for TaqMan detection, one aimed at the δ-*Proteobacteria* and one directed specifically at *Geobacter.* For δ-*Proteobacteria*, we utilized primers 361F and 685R (Table 1), where the 3′-terminal adenines in 361F are contiguous with the 5′ adenines in primer S-δ401F-20. Primer 685R differs from primer S-δ683aR-20 by only one base. *Geobacter*-specific PCR was achieved with primers 561F and 825R.

Internal fluorogenic probes targeted a more general eubacterial sequence and a *Geobacter*-specific sequence within the 16S rRNA and were designed with Primer Express 1.0 software (Perkin-Elmer) and the recommended guidelines for TaqMan probe design. TaqMan probes were obtained from Perkin-Elmer, labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) and 6-carboxytetramethyl rhodamine (TAMRA), as listed in Table 1. TaqMan probes Gbc1 and Gbc2 are specific for *Geobacter*, whereas probe Eub1 is complementary to a broad range of eubacterial 16S rRNAs (including *Geobacter*).

**TaqMan PCR optimization.** TaqMan PCR conditions must be empirically determined for each primer-probe combination. We therefore followed Perkin-Elmer guidelines, performing PCRs with optical-grade 96-well thermocycling plates, 50 μl of total reaction mixture volume, and 5 μl of target DNA or 2 μl of cDNA reaction products. The TaqMan reaction buffer contained 5.5 mM $MgCl_2$; 200 nM each dATP, dCTP, and dGTP; 400 nM dUTP, 0.5 U of uracyl DNA glycosylase, and 1.25 U of AmpliTaq gold. TaqMan probe concentrations were maintained at 100 nM, while PCR primer concentrations were systematically varied in all pairwise combinations between 50 and 900 nM for both the forward and reverse primers. PCR amplification and detection for all primer-probe combinations were performed with the ABI 7700 Sequence Detection system with 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 s and 55°C for 60 s. Optimum concentrations of TaqMan PCR primers are reported in Results and appropriate figure and table legends.

**TaqMan quantitation.** External standards were generated from known quantities of *G. chapellei* and "*G. bemidjiensis*" genomic DNA or 16S rRNA, spanning 6 orders of magnitude (from $5 \times 10^0$ to $5 \times 10^6$ copies). The detection threshold was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15. Standard curves relating the threshold cycle ($C_t$) to DNA or RNA concentrations were generated with ABI Prism 7700 software (Perkin-Elmer).

## RESULTS

**TaqMan optimization for 16S rDNA and rRNA.** Two primer sets and three probes were developed to address several questions related to the abundance, distribution, and activity of metal-reducing bacteria in pristine and contaminated subsurface environments. Primers 361F, 561F, and 825R are specific for *Geobacter* and very closely related isolates (as determined by comparison against the Ribosomal Database Project [28]) and were originally designed from the 16S rRNA of *G. chapellei*. Primer 685R is complementary to many iron- and sulfate-reducing genera within the δ-*Proteobacteria*, including *Geobacter*, *Pelobacter* (including fermentative species), *Desulfovibrio, Desulfomicrobium, Desulfuromusa,* and *Desulfuromonas* (including dissimilatory S reducers). Three specific TaqMan assays were developed for both DNA and RNA templates, with standard curves and performance specifications illustrated in Fig. 1. Assay 1A is directed at known *Geobacter* species as represented in the Ribosomal Database Project, with the potential to amplify related species (including primer 685R). Assay 1B was designed to further detect and quantify unknown *Geobacter* spp. or close relatives by incorporating the broad-spectrum detector probe Eub1. Finally, assay 1C is specific for "*G. bemidjiensis*," a new environmental isolate that is very closely related to unculturable, Fe(III)-reducing *Geobacter* spp. that are easily stimulated in sandy aquifer sediments (41).

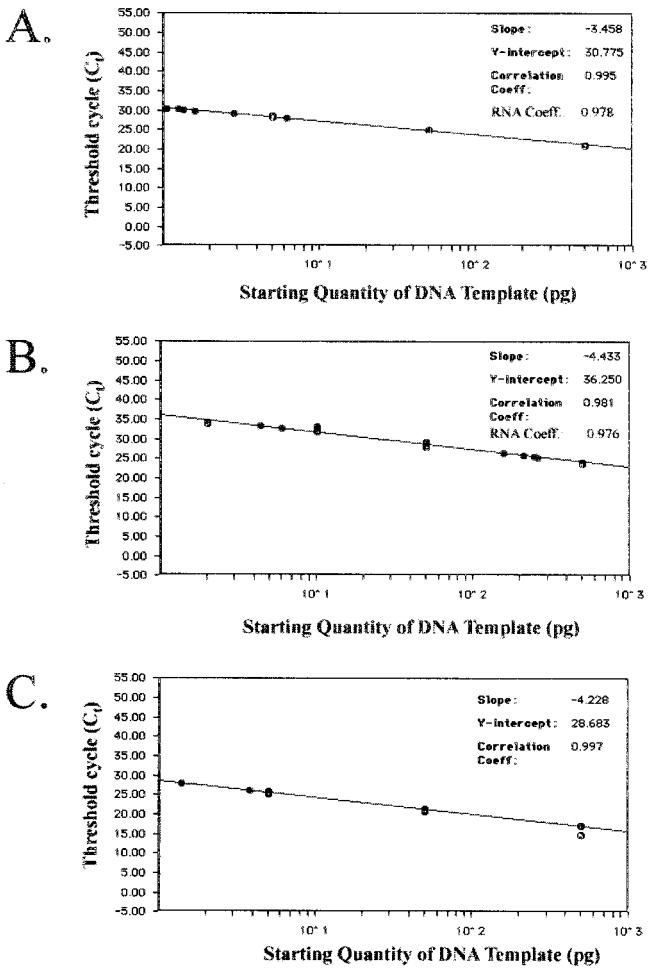


FIG. 1. TaqMan standard curves for *Geobacter* DNA templates. Coefficients of variation for RNA standard curves are provided in the upper right of the panels. Assays 1A (A), 1B (B) and 1C (C) were carried out as described in the text. An RNA standard curve and coefficient of variation were not tested for assay C, specific for "*G. bemidjiensis*."

Each TaqMan assay was originally developed using 100 pg of genomic DNA or 50 pg of 16S rRNA from *G. chapellei* and/or "*G. bemidjiensis*" to optimize primer-probe concentrations and target specificity. Optimum primer and probe concentrations for assays 1A and 1B were 900 nM for 361F, 300 nM for 685R, and 100 nM TaqMan probe Gbc1 or Eub1. Assay 1C required 300 nM each of 561F and 825R and 100 nM of probe Gbc2. All optimized assays utilized a two-step cycling regime consisting of 45 cycles at 95°C for 15 s and 55°C for 60 s. Agarose gel electrophoresis of TaqMan reaction mixtures consistently showed discrete PCR products of the expected molecular weight for all assays (not shown). The lower detection limit of each assay was 5 to 50 fg of genomic DNA or ≤2 pg of 16S rRNA. Results in Fig. 1 show that the correlation coefficients for DNA standard curves were higher than RNA standard curves, a consistent result that we attribute to uncontrolled

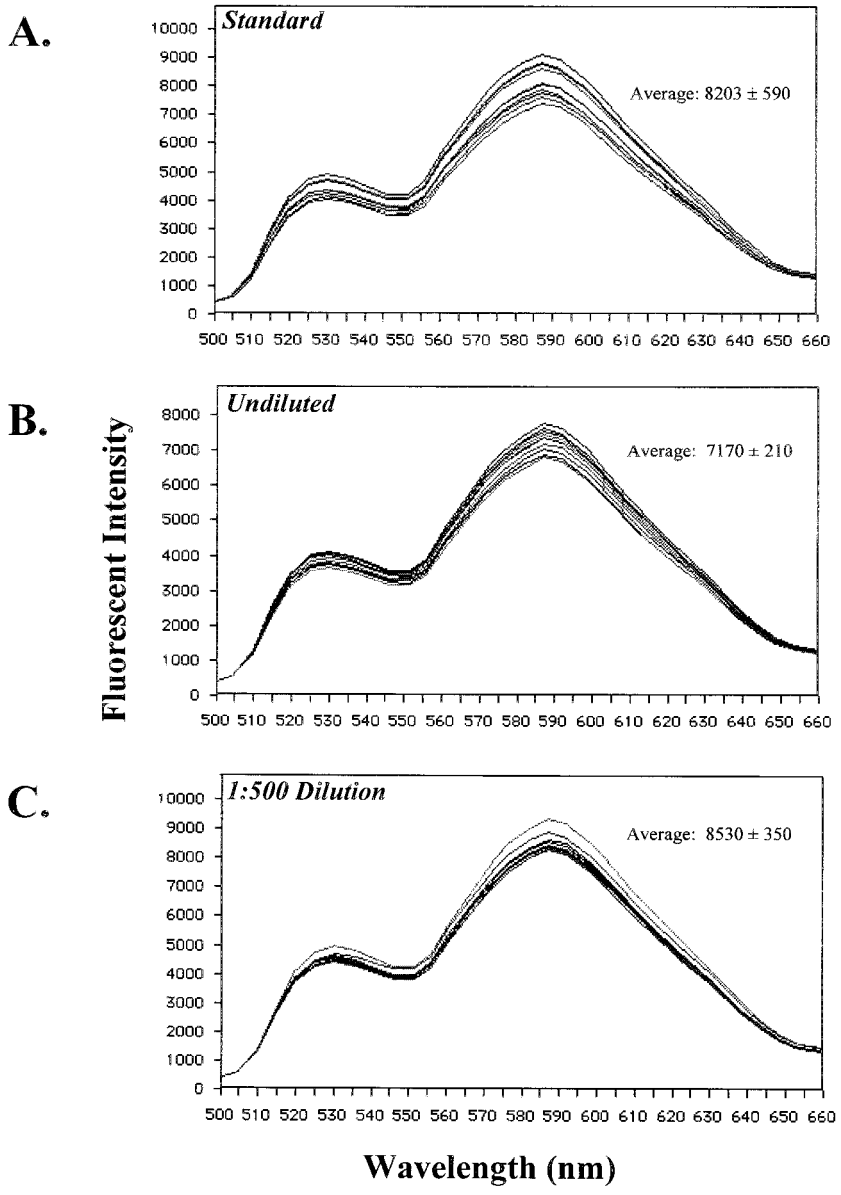


FIG. 2. Raw spectral traces for "*G. bemidjiensis* " cells amended into sterile sediment and analyzed with assay 1B. The traces represent the unprocessed fluorescence trace for all of the reporter fluorescents within the TaqMan assay (i.e., FAM, TAMRA, and ROX) before thermal cycling was initiated. All samples were amplified on the same day in the same 96-well plate, such that the scale of fluorescent emission for each of the panels is directly comparable. (A) External standard curve of DNA isolated from pure culture; (B) undiluted sediment extract; and (C) sediment extract diluted 500 times prior to analysis. Average, average peak intensity ($n = 10$) at the TAMRA quencher emission maximum (590 nm). The statistically significant decrease in TAMRA quencher emission between undiluted and diluted samples provided the first indication of fluorescence quenching in the TaqMan PCR system.

variability in RT efficiency. Likewise, the correlation coefficients frequently dropped below the $R^2 = 0.99$ level recommended by Perkin-Elmer for precise and accurate quantitation, especially for RNA standard curves.

**Amended sediment studies.** Sterilized, uncontaminated aquifer sediments were amended with $2.9 \times 10^5$ "*G. bemidjiensis*" cells and analyzed with TaqMan assay 1C. Our initial observation was that unprocessed fluorescence traces (inclusive of FAM, TAMRA and carboxy-X-rhodamine [ROX] emissions) from PCR tubes containing undiluted sediment extracts were consistently lower than the unprocessed fluorescence traces for the external standard curve by approximately 1,000 relative units throughout the entire PCR run (Fig. 2A and B). The observed difference in mean emission intensity was, in fact, statistically significant ($P < 0.0002$; Student's $t$ test). There was a similar, significant decrease in mean quencher emission for undiluted extracts relative to diluted extracts ($P < 0.0001$), but the standard curve and 1:500 diluted extracts showed no difference at the 95% confidence level ($P > 0.16$).

Despite fluorescence quenching due to copurified contaminants, the precision or uniformity of the raw data traces in

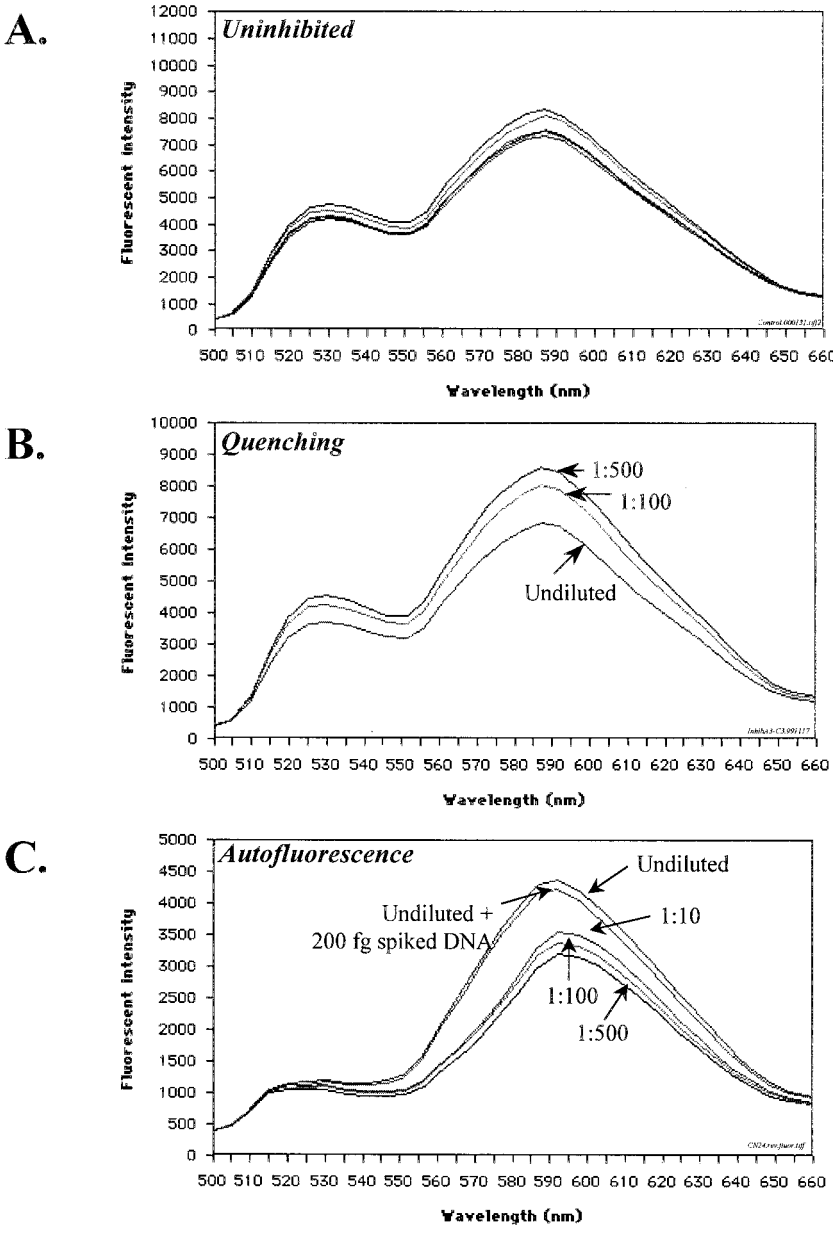


FIG. 3. Fluorescence quenching and autofluorescence in environmental nucleic acid extracts. Panel C represents a different sediment sample, analyzed with a different 96-well plate. Consequently, the scale of the fluorescent emission is not directly comparable to the scales shown in panels A and B. (A) Uninhibited trace from the external standard curve; (B) fluorescence quenching from amended Bemidji aquifer sediment extract; and (C) autofluorescence from Cerro Negro subsurface sediment extract.

undiluted or diluted sediment extracts was nevertheless similar to the external standard curve (Fig. 2). For all seeded sediments ($n = 10$), the coefficient of variation (CV) around the 590-nm peak emission was 7.2% for the external standard curve (Fig. 2A), 2.9% for undiluted extracts (Fig. 2B), and 4.1% for diluted extracts (Fig. 2C). The discrepancy in raw data traces and its implications for PCR accuracy are nevertheless evident in the results shown in Fig. 3A and B. In this sample, the quencher CV in the uninhibited standard curve was 3.3%, whereas the CV over all dilution levels of the test sediment template was 11.3%. The resulting TaqMan quantitation on undiluted extracts (Fig. 3B) was 1 order of magnitude lower than either 1:100 or 1:500 template dilution, whereas limiting-dilution PCR resulted in estimates of the same order of magnitude regardless of template dilution (not shown). If PCR enumerations were based solely upon the undiluted sample eluant, the TaqMan assay generated an inaccurate result even though the $C_t$ measurements were very precise and reproducible in the sediment extracts (Fig. 2).

Applying the 361F-685R-Eub1 TaqMan assay to DNA quantitation in Cerro Negro sediments, we also obtained evidence for autofluorescence in undiluted extracts (Fig. 3C). Similar results (quenching and autofluorescence) were also observed with other sediments from Bemidji and Cerro Negro

TABLE 2. TaqMan estimates of cell copy number for diluted and undiluted sediment extracts[a]

| Dilution | Estimates of seeded cell copy number ($\log_{10}$)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7.47 | 6.47 | 5.47 | 4.47 | 3.47 | 2.47 | 1.47 |
| Full strength | No reading | 1.92 | 2.69 | 1.63 | 2.04 | 1.20 | 0.68 |
| 1:100 | 7.72 | 5.58 | 4.99 | 4.04 | 3.46 | 2.73 | 1.28 |
| 1:500 | 7.93 | 6.58 | 5.66 | 4.85 | 3.74 | 2.53 | 2.95 |

[a] Estimates represent single-point values from one seeded sediment, one extraction, and one point in the dilution series. Consequently, there are no standard errors for these data.

[b] Sterile sandy sediment was seeded with $2.94 \times 10^7$ to $<1 \times 10^0$ "*G. bemidjiensis*" cells $g^{-1}$ and extracted with the BIO 101 reagent as described in the text. TaqMan quantitation utilized assay 1C, with primers 561F-825R and probe Gbc2 (specific for "*G. bemidjiensis*"). All seeded sediments with $\leq 10^0$ cells $g^{-1}$ gave no amplification signal after 45 cycles.

sampling sites, while some sediments used in this study showed no evidence of fluorescence quenching or autofluorescence. Thus, the incidence, extent, and effect of humic acid quenching or autofluorescence for any sample cannot be deduced a priori and varies from sample to sample in an unpredictable manner.

"*G. bemidjiensis*" was added to uncontaminated aquifer sediment (from $2.94 \times 10^7$ to 2.94 cells $g^{-1}$) and analyzed with TaqMan assay 1C. Assuming similar (≥10%) extraction efficiency for all sediment samples and one 16S rDNA copy per genome, TaqMan quantitation in undiluted extracts underestimated the cell concentration by up to 5 orders of magnitude (Table 2) even though there was no PCR inhibition based on gel electrophoresis of amplification products (not shown). Interestingly, the undiluted extracts gave approximately the same estimate of seeded cell concentration ($10^1$ to $10^2$ cells) regardless of the initial seeded cell number. Diluted (1:500) templates, however, gave TaqMan estimates of the same order of magnitude as the seeded cell concentration. These results also support the hypothesis that TaqMan inaccuracy resulted from fluorescence quenching rather than PCR inhibition.

TaqMan inaccuracy was mitigated by adopting a replicate limiting-dilution format (6). As shown in Table 3, averaging TaqMan estimates of DNA concentration (or cell number) over multiple extracts and multiple dilutions produced a much more robust and accurate estimate of starting cell concentration, even when full-strength sediment extracts (Table 2) were included in the calculation. Excluding the full-strength (e.g., obviously quenched) enumerations from the data set resulted in significantly different estimates of cell concentration for some of the seeded sands, but not the seeded aquifer sediments (Table 3). Because the effects of fluorescence quenching are variable and cannot be anticipated a priori, we therefore encourage the use of multiple dilutions and/or extracts for TaqMan quantitation in sediments and other samples where humic acids or other contaminants are known to copurify with target nucleic acids. In particular, we emphasize the importance of performing a dilution series, more so than performing replicate analyses at a single dilution level (especially undiluted extracts) (Table 2).

**RNA quantitation in unseeded sediments.** Successful quantitation of rDNA in seeded sediments led to the evaluation of TaqMan PCR for rRNA quantitation in unamended sediment samples. Uncontaminated Bemidji aquifer sediments were stimulated for Fe reduction through the addition of formate. Duplicate 16S rRNA extractions were performed, and duplicate rRNA dilution series were generated from each extract as described above. Diluted rRNA templates were converted to cDNA, and TaqMan assays 1A (assaying known *Geobacter)* and 1B (i.e., assaying known and unknown *Geobacter* spp.) were applied to quantify cDNA transcripts. In the absence of an external quantification method for native *Geobacter* spp., we utilized the limiting-dilution qPCR method to quantify cDNA transcripts and provide a basis for evaluating TaqMan performance.

Results for both TaqMan assays are shown in Table 4. Background levels of *Geobacter* spp. rRNA, as measured by limiting-dilution PCR, were $1.0 \times 10^4$ pg ($\log_{10} = 4.00$) of 16S rRNA g of sediment$^{-1}$. As with the rDNA analysis, the undiluted extracts showed evidence of fluorescence quenching in the TaqMan calculation of 16S rRNA quantities. In this case, excluding the undiluted extracts from the estimates of rRNA abundance led to significantly different conclusions for the two TaqMan assays, even though a comparison of all data and the 1:100 and 1:500 dilution enumerations were statistically similar ($P = 0.331$ and 0.372 for assays 1A and 1B, respectively). We

TABLE 3. Effect of fluorescence quenching on TaqMan quantitation of seeded sediments[a]

| Seeded cell number[b] | $\log_{10}$ value | Sand sediment | | | Aquifer | | |
|---|---|---|---|---|---|---|---|
| | | All data[c] | 1:100 + 1:500 dilutions[c] | *t* test *P* value[d] | All data[c] | 1:100 + 1:500 dilutions[c] | *t* test *P* value[d] |
| $2.9 \times 10^7$ | 7.46 | Quenching | 7.83 ± 0.11 | ND | Quenching | 7.72 ± 0.15 | ND |
| $2.9 \times 10^6$ | 6.46 | 5.26 ± 1.76 | 5.98 ± 1.12 | 0.397 | 6.43 ± 0.62 | 6.83 ± 0.15 | 0.147 |
| $2.9 \times 10^5$ | 5.46 | 4.70 ± 1.25 | 5.50 ± 0.33 | 0.154 | 5.18 ± 0.63 | 5.56 ± 0.29 | 0.193 |
| $2.9 \times 10^4$ | 4.46 | 3.76 ± 1.40 | 4.62 ± 0.36 | 0.165 | 4.53 ± 0.61 | 4.92 ± 0.17 | 0.152 |
| $2.9 \times 10^3$ | 3.46 | 3.27 ± 0.74 | 3.74 ± 0.21 | 0.160 | 3.36 ± 0.74 | 3.71 ± 0.30 | 0.203 |
| $2.9 \times 10^2$ | 2.46 | 2.38 ± 0.73 | 2.85 ± 0.21 | 0.159 | 2.38 ± 0.73 | 2.98 ± 0.27 | 0.217 |
| $2.9 \times 10^1$ | 1.46 | 1.52 ± 0.94 | 2.14 ± 0.69 | 0.284 | 1.52 ± 0.94 | 2.53 ± 0.36 | 0.090 |

[a] Fine-grained pure quartz sand and Bemidji aquifer sediments were sterilized by being autoclaved for 1 h and then exposed to 260-nm UV light for 1 h before being seeded with a known quantity of "*G. bemidjiensis*". TaqMan quantitation utilized assay 1C (primers 561F-825R and probe Gbc2).

[b] Cell concentrations were determined by acridine orange direct counts.

[c] A single-seeded sand or aquifer sediment was extracted in triplicate, and a single dilution series (undiluted, 1:100, and 1:500) was generated from each extract. TaqMan estimates of 16S rRNA abundance were log transformed, and a geometric mean was calculated (± standard deviation). The TaqMan estimates here therefore represent nine individual TaqMan datum points (three extracts times three dilutions). All seeded sediments of $\leq 10^1$ cells $g^{-1}$ gave no amplification signal after 45 cycles.

[d] A two-tailed Student's *t* test was applied to the enumerations for all data or the restricted data set for the sand and aquifer sediments, respectively. *P* values are given for a 95% confidence level. ND, not determined.

TABLE 4. TaqMan and limiting-dilution qPCR for Geobacter 16S rRNA in stimulated sediments[a]

| Sample | TaqMan[b] | | | | Limiting dilution[c] |
|---|---|---|---|---|---|
| | All data | | 1:100 and 1:500 dilutions | | |
| | Assay 1A[d,e] | Assay 1B[d] | Assay 1A[d,e] | Assay 1B[d] | |
| Dilution Series 1 | 3.90 ± 0.23 | 4.03 ± 0.22 | 4.01 ± 0.18 | 4.14 ± 0.16 | 6.45 |
| Dilution Series 2 | 3.93 ± 0.19 | 4.19 ± 0.24 | 4.04 ± 0.03 | 4.32 ± 0.08 | 6.80 |
| Average | 3.92 ± 0.19 | 3.90 ± 0.23 | 4.03 ± 0.11 | 4.23 ± 0.15 | 6.62 ± 0.25 |

[a] Uncontaminated Bemidji aquifer sediments (40 g [dry weight]) were amended with 5 mM (final concentration in 40 g) formate. Only one of the triplicate microcosm experiments was sampled and used for the data presented here. Background levels of *Geobacter* spp. in nonamended sediments were approximately $1.0 \times 10^4$ pg of 16S rRNA $g^{-1}$ ($\log_{10} = 4.00$) as determined by limiting-dilution PCR. Values are $\log_{10}$ (picograms of 16S rRNA per gram).

[b] For TaqMan quantitations, a single 0.5-g aliquot was extracted, and two independent dilution series were prepared (undiluted, 1:100, and 1:500). The average values for TaqMan PCR therefore represent six datum points. Data was analyzed as in Table 3, either with (all data) or without (1:100 and 1:500) the undiluted (i.e., quenched) extract.

[c] For replicate limiting-dilution analyses, two 0.5-g aliquots of sediment were extracted, and two independent dilution series were generated from each extract. PCR results are therefore the average values for a $2 \times 2$ analytical matrix.

[d] A two-tailed Student's *t* test at the 95% confidence level for the two dilution series within a TaqMan assay (assay 1A and 1B) was done. *P* values for all data were 0.727 (assay 1A) and 0.047 (1B); for the 1:100 and 1:500 dilutions, *P* values were 0.855 (1A) and 0.168 (1B).

[e] A two-tailed Student's *t* test at the 95% confidence level across the two TaqMan assays was done, taking into account both dilutions within an assay. The *P* values for all data were 0.042 but 0.162 for the 1:100 and 1:500 dilutions.

conclude from these results that the number of replications is just as important for accurate TaqMan enumeration as performing a dilution series, as fluorescence quenching in undiluted extracts had a more profound influence on TaqMan enumeration when only two dilution series were prepared and analyzed (Table 4) rather than three dilution series (Table 3). By excluding the data from undiluted extracts, statistically similar TaqMan 16S rRNA enumerations were obtained for both TaqMan assays. However, the limiting-dilution PCR technique resulted in a 2 to 3 log increase in 16S rRNA enumeration for the identical sediment extracts. While we cannot (yet) statistically compare the values obtained from TaqMan and limiting-dilution PCR, we believe that the 2 to 3 log difference in 16S rRNA estimates probably has some practical significance for biological interpretations of *Geobacter* sp. abundance and activity in these sediments.

## DISCUSSION

**TaqMan advantages.** TaqMan PCR has a number of perceived advantages over competitive and/or MPN qPCR techniques, principally in detection sensitivity, speed, and dynamic range (36). By using a threshold cycle ($C_t$) rather than a direct measure of PCR product abundance, TaqMan also involves a fundamentally different measurement and data interpolation than do conventional techniques. An important consequence of the $C_t$ measurement scheme is reduced CV around the detection measurement. For example, Desjardin et al. (11) reported that the TaqMan assay resulted in only 10% CV for target enumeration in test samples, whereas duplicate cPCR tests resulted in 74 and 98% CV. Because of these benefits, TaqMan assays are under continued development for detecting microorganisms in both clinical and food safety arenas (2, 9, 11, 14, 15, 23, 29, 32, 34, 35, 40, 49), with recent applications to aqueous environments (4, 42, 43).

**Fluorescence quenching and autofluorescence.** It is well known that PCR inhibitors in environmental samples affect PCR and amplification efficiency (45, 48). $C_t$ measurements are also dependent upon the starting template copy number, DNA amplification efficiency, and efficiency of TaqMan probe cleavage (17, 26, 36). For the TaqMan assay, however, issues of PCR inhibitors extend beyond the PCR itself and into the detection method, because accurate and precise $C_t$ determinations are also dependent upon perfect (or uninhibited) performance and detection of the reporter fluors. In this vein, technical details related to fluorescence detection are normally discussed within the context of FRET and the placement of quencher-reporter fluors on the TaqMan probe (e.g., in the ABI PRISM 7700 user's manual), rather than the detection of fluorescence emissions in a contaminated background.

Fluorescence detection therefore presents a significant challenge for routine analysis of soils or sediments, since humic acids can either quench or autofluoresce at the excitation-emission wavelengths of common fluors (FAM, TAMRA). Whether the quenching or autofluorescence affects the quencher or reporter is of little practical consequence, because the dependence upon FRET for signal generation inextricably links both fluors. That is, changes in energy absorption or emission of the quencher will necessarily affect the reporter. This result and its consequences on qPCR accuracy were discovered during the application of TaqMan PCR for DNA quantitation in seeded sediments and are exemplified in the raw (unprocessed) data traces shown in Fig. 2.

The consequence of fluorescence quenching is to increase $C_t$ and underestimate the starting target concentration in the original sample. The consequence of autofluorescence is to decrease $C_t$ and overestimate the starting target concentration in the original sample, irrespective of assay precision. Clearly, a consistent and statistically significant downward (or upward) shift in quencher or reporter fluorescence intensity in undiluted environmental extracts will preclude accurate target quantitation in true unknowns. This conclusion was supported during quantitative analysis of amended sediments (Table 2). As a consequence of the observed fluorescence quenching and/or autofluorescence in sediment extracts, we therefore conclude that the TaqMan assays reported here ($R^2 \geq 0.97$) are sufficiently precise for most ecological investigations of metal reducer distribution, abundance, and activity. We further contend that a requirement for standard curves with $R^2 >$

0.99 (as opposed to $R^2 > 0.95$, for example) has little meaning for the quantitation of sediment unknowns.

**RNA quantitation in native sediments.** 16S rRNA can be considered a rough indicator of general microbial activity or viability in the environment, whereas mRNA is generally considered an indicator of specific metabolic activity. A significant question that arises from the 16S rRNA quantitation in native sediments (Table 4) is whether TaqMan PCR is generating an accurate estimate of 16S rRNA abundance or if the TaqMan and limiting-dilution qPCR are truly measuring the same biological entity.

It is possible that the TaqMan value is overly affected by fluorescence quenching, especially when the undiluted extracts were used in the TaqMan calculation. However, statistical analysis showed statistically similar TaqMan enumerations (within an assay) when the undiluted extract was included or excluded from the calculation. Further, we saw no obvious quenching effects in the raw data traces from the diluted samples (not shown).

It is also possible that the TaqMan detector probes are too narrow in phylogenetic breadth to allow for a fair comparison between the two PCR methods, even though assay 1B utilized a broad-spectrum eubacterial detector probe. That is, the TaqMan detector probe provides a third level of specificity to the PCR assay before DNA is detected, whereas limiting-dilution PCR only has two levels of specificity (the two PCR primers). Because we do not know the extent of cross-reactivity between our PCR primers and the rRNA from indigenous (unknown) bacteria or between the detector probe and indigenous species, it is possible that the TaqMan assay does not detect the same population of *Geobacter* spp. as a simple PCR. Alternatively, the detector probe may be much more sensitive to single base mismatches or humic acid interactions with target DNA than are PCR primers and the amplification process per se. Testing this hypothesis will require the design of new detector probes with various mismatches (number and position) and a better understanding of humic acid interaction with native (unamended) nucleic acids. We cautiously conclude, however, that the two qPCR methods are measuring different aspects of the microbial population and are not directly comparable.

**Summary.** A 10-fold difference in qPCR estimates may have little to no ecological or biological significance, as in the detection of metal-reducing bacteria in subsurface sediments. For pathogen surveillance and monitoring, however, a 10-fold difference in qPCR estimates may have profound biological (and practical) significance. Whether or not fluorescence quenching affects assay accuracy and performance criteria further depends upon the extent of fluorescence quenching and/or autofluorescence, humic acid contamination, nucleic acid extraction efficiency, PCR inhibition, target copy number per organism, and numerous other variables. Our results clearly demonstrate that fluorescence quenching and autofluorescence can significantly affect TaqMan PCR enumeration accuracy, with subsequent implications for the design and implementation of TaqMan PCR to sediments and related environmental samples. However, by employing a system of replicate dilutions and replicate analyses, we have demonstrated that TaqMan PCR can still accurately quantitate nucleic acids in sediments.

To account for and minimize the effects of fluorescence quenching on assay accuracy and performance, we therefore recommend adopting many of the features of the replicate limiting-dilution method (6) in the application of TaqMan PCR. That is, we now routinely perform multiple nucleic acid extracts from each sample, and use multiple 3 log template dilutions for each extract, yielding from 6 to 12 independent measures of DNA or RNA abundance for each sample. Similar strategies must be embodied in the automated assays of advanced instrumentation (5, 22, 33, 44) if they are likewise to have practical utility for the real-time detection of microorganisms in sediments and related environmental samples. For TaqMan PCR, however, the inclusion of amended controls is probably not necessary, as the extent of autofluorescence and/or quenching on reporter fluorescence cannot be calculated from ABI PRISM 7700 data alone. Further, a decrease or increase in TAMRA emissions can be ascertained directly from the raw spectral traces of the standard curve and unknowns.

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