Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the Dehalococcoides 16S rRNA gene in groundwater

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ABSTRACT
Quantitative real-time PCR (qPCR) commonly uses the fluorogenic 5¢ nuclease (TaqMan) and SYBR Green I (SG) detection chemistries to enumerate biomarker genes. Dehalococcoides (Dhc) are key bacteria for the detoxification of chlorinated ethenes, and the Dhc 16S ribosomal RNA (rRNA) gene serves as a biomarker for monitoring reductive dechlorination in contaminated aquifers. qPCR enumeration of Dhc biomarker genes using the TaqMan or SG approach with the same primer set yielded linear calibration curves over a seven orders of magnitude range with similar amplification efficiencies. The TaqMan assay discriminates specific from nonspecific amplification observed at low template concentrations with the SG assay, and had a 10-fold lower limit of detection of ~3 copies per assay. When applied to Dhc pure cultures and Dhc-containing consortia, both detection methods enumerated Dhc biomarker genes with differences not exceeding 3-fold. Greater variability was observed with groundwater samples, and the SG chemistry produced false-positive results or yielded up to 6-fold higher biomarker gene abundances compared to the TaqMan method. In most cases, the apparent error associated with SG detection resulted from quantification of nonspecific amplification products and was more pronounced with groundwater samples that had low biomarker concentrations or contained PCR inhibitors. Correction of the apparent error using post-amplification melting curve analysis produced 2 to 21-fold lower abundance estimates; however, gel electrophoretic analysis of amplicons demonstrated that melting curve analysis was insufficient to recognize all nonspecific amplification. Upon exclusion of nonspecific amplification products identified by combined melting curve and electrophoretic amplicon analyses, the SG method produced false-negative results compared to the TaqMan method. To achieve sensitive and accurate quantification of Dhc biomarker genes in environmental samples (e.g., groundwater) and avoid erroneous conclusions, the analysis should rely on TaqMan detection chemistry, unless additional analyses validate the results obtained with the SG approach.

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1. Introduction
Excessive use of tetrachloroethene (PCE) and trichloroethene (TCE) as dry cleaning and metal degreasing agents, respectively, resulted in widespread aquifer contamination, where abiotic and biotic processes lead to the formation of dichloroethenes (DCEs) and vinyl chloride (VC) (see U.S. Environmental Protection Agency Superfund Site Information at http://www.epa.gov/superfund/sites/siteinfo.htm; Moran et al., 2007). Under anoxic conditions, diverse groups of bacteria contribute to PCE and TCE reductive dechlorination to cis-1,2-DCE (reviewed in Smidt and de Vos, 2004), but only members of the Dhc group are capable of complete reductive dechlorination of cis-DCE and VC to ethene (Cupples et al., 2003; He et al., 2003b; Maymó-Gatell et al., 1997; Sung et al., 2006). Several studies established a link between the presence of Dhc and ethene formation at contaminated sites (Fennell et al., 2004; Hendrickson et al., 2002; Lendvay et al., 2003; Major et al., 2002; van der Zaan et al., 2010). Therefore, the accurate identification and quantification of Dhc biomarker genes has become increasingly important for site assessment and the implementation of bioremediation using naturally occurring bacteria in the treatment and cleanup of sites impacted by chlorinated pollutants.

Quantitative real-time PCR (qPCR) has emerged as the method of choice for environmental monitoring of Dhc biomarker genes. To this end, several groups have developed qPCR primers that target the Dhc 16S rRNA gene (Cupples et al., 2008; He et al., 2003a; Ritalahti et al., 2006). The known Dhc genomes harbor a single copy of the 16S rRNA gene indicating that the enumeration of this target allows for estimates of Dhc cell abundance. qPCR enumeration of the Dhc 16S rRNA gene established a relationship between Dhc population abundances and remedial...
success (i.e., ethene formation and detoxification) [Lee et al., 2008; Lendvay et al., 2003] and is useful to predict dechlorinating potential at contaminated sites (Lendvay et al., 2003; Rahm et al., 2006; Ritalahti et al., 2010a).

The two most commonly used detection chemistries for qPCR assays are the TaqMan chemistry and the SG chemistry. When surveyed in 2005, the TaqMan assay was the most used (72%) (Bustin, 2005) but a later survey estimated that the SG chemistry gained ground and was used in 85% of qPCR assays (Colborn et al., 2008). The ease and somewhat lower cost of qPCR assays using the SG chemistry compared to the TaqMan method, which requires the design and purchase of a fluorogenic, linear hybridization probe, are most often cited as the reasons for favored usage of the SG method.

The SG approach uses two PCR primers and the SG dye that is included in the amplification mix. The SG dye binds nonspecifically to all double-stranded nucleic acid molecules including specific and nonspecific product generated during PCR, as well as primer-dimers (Higuchi et al., 1993; Wittwer et al., 1997). For the TaqMan assay, target gene quantification not only requires the two amplification primers, but also a fluorogenic probe binding between the amplification primers. Following target amplification, the TaqMan probe anneals to the single-stranded amplicon. The probe is then displaced and cleaved by the 3′ → 5′ exonuclease activity of the Taq polymerase during the next round of amplification. The fluorescent signal is thus produced by the release of the fluorophore on the 5′ end of the probe from the quencher on the 3′ end (Holland et al., 1991; Wittwer et al., 1997). Only amplicons to which the TaqMan probe hybridizes contribute to the fluorescent signal. While qPCR assays utilizing the SG chemistry can rival the sensitivity of the TaqMan method, a distinct advantage of the TaqMan chemistry is higher target specificity due to the requirement of a matching probe binding between the amplification primers (Newby et al., 2003; Wittwer et al., 1997).

Assays for the enumeration of Dhc 16S rRNA biomarker gene utilize both the TaqMan and SG detection chemistries. While laboratory results demonstrate that both assays produce comparable results with template DNA from defined samples (e.g., pure culture DNA), their application to field samples has not been systematically evaluated. In this study, the direct comparison of both detection chemistries for the quantification of the Dhc 16S rRNA biomarker gene from laboratory and groundwater samples using a single primer set was undertaken. The effects of qPCR assay detection chemistry on Dhc biomarker gene abundance estimation and subsequent bioremediation decision-making are discussed.

2. Methods

2.1. Samples

DNA was obtained from Dhc sp. strain BAV1, the PCE-to-ethene-dechlorinating consortium KB-1, which contains two Dhc strains, and groundwater samples collected from chlorinated ethene-contaminated sites. For collection of biomass, 5 mL of Dhc sp. strain BAV1 culture was passed through a 25 mm 0.22 μm pore size Durapore hydrophilic polyvinylidene fluoride membrane (Millipore, Billerica, MA) by vacuum filtration. Dilutions of consortium KB-1 with cell titers in the range of 1.0 × 10^7 to 1.0 × 10^8 Dhc cells per L were obtained by L. Edwards, University of Toronto. Each polypropylene vessel contained 570 mL of KB-1 culture suspension and was shipped in a cooler on blue ice via overnight carrier. Upon receipt, the biomass was collected by vacuum filtration onto a 0.22 μm pore size, 47 mm diameter polystyrene sulfone (PES) membrane (MO BIO Laboratories, Carlsbad, CA). Contaminated groundwater samples were obtained from Vandenberg Air Force Base, CA (3 wells) and the Bachman Road site in Oscoda, MI (11 wells), and Sterivex cartridges were received from Naval Air Station at Cecil Field, Site 59, FL (9 wells) and Ft. Dix Army Base, Ft. Dix, NJ (9 wells). Groundwater and Sterivex cartridges were shipped by overnight carrier on blue ice. Upon receipt, the biomass was collected from groundwater samples by vacuum filtration onto a 0.22 μm pore size, 47 mm diameter PES membrane and immediately stored at −80 °C prior to DNA isolation. On site biomass collection used Sterivex-GP cartridge filters (Millipore, Billerica, MA, Catalog #SVEPL10RC) and was performed as described (Ritalahti et al., 2010a). Filters were stored at −80 °C prior to DNA isolation.

2.2. DNA isolation from membrane filters

Total DNA was isolated from the frozen MO BIO filters using the UltraClean Water DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer’s instructions or directly from the frozen Durapore or Sterivex filters by addition of the filter to the PowerSoil DNA Isolation Kit bead tubes (MO BIO Laboratories, Carlsbad, CA) and proceeding according to the manufacturer’s instructions (Ritalahti et al., 2010a,b). DNA from the UltraClean Water Isolation Kit was eluted into a final volume of 3 mL of elution buffer (10 mM Tris, pH 8.5) and concentrated by addition of 1/10 volume of 5 M NaCl, 2 volumes of absolute, ice cold ethanol, and incubation for 30 min at 4 °C. The DNA was collected by centrifugation (13,200 rpm, 15 min, room temperature), rinsed with 70% (v/v) ethanol, air dried and suspended in a final volume of 100 μL elution buffer. Total DNA isolated using the PowerSoil DNA isolation kit was recovered in 100 μL elution buffer. DNA concentrations were determined by absorption readings at 260 nm using a NanoDrop Spectrophotometer ND-1000 and DNA quality was inferred by the 260/280 absorbance ratios.

2.3. qPCR primers and TaqMan probe

The primer set targeting a Dhc 16S rRNA gene sequence was previously described and validated, and generated a 66-basepair amplicon (He et al., 2003a; Ritalahti et al., 2006). The TaqMan probe for the Dhc 16S rRNA gene carried the 5′ fluorescent dye 6-carboxyfluorescein (FAM) and the 3′ Black Hole-1 quencher instead of the TAMRA quencher (He et al., 2003a; Ritalahti et al., 2006). Primers and probe were purchased from Integrated DNA Technologies (San Diego, CA). All qPCR assays used a 300 nM concentration of each forward and reverse primer, with the TaqMan assay also receiving a 300 nM concentration of the linear hybridization probe.

2.4. TaqMan assays

All TaqMan assays used the TaqMan universal PCR master mix from Applied Biosystems. In a total volume of 20 μL, each reaction contained 1× PCR master mix, primers, probe and 2 μL template DNA. Three qPCR tubes received undiluted template DNA and three tubes received 1:10 diluted template DNA. This resulted in six quantifications per DNA sample and allowed for a determination of the presence of PCR inhibitors in the DNA preparations. Because PCR assays are easily contaminated, the master mix contained a mixture of dTTP/dUTP nucleotides and the AmpErase uracil N-glycosylase (UNG) (Applied Biosystems) as a safeguard against contamination by amplification products from previously run reactions. Prior to amplification, the reactions were heated for 2 min at 50 °C to activate the AmpErase UNG to hydrolyze the N-glycosidic bond between the uracil base and the deoxyribose backbone from carry-over products and prevent their use as templates in the current reaction. Following a 10 min denaturation step at 94 °C, amplification was carried out for 40 cycles of 15 s at 94 °C and 1 min at 60 °C in the presence of the TaqMan probe. The fluorescence increase due to DNA amplification at any given cycle within the exponential phase of PCR is proportional to the initial number of template copies. The number of PCR cycles (i.e., the Ct or the threshold cycle) needed for the amplification
curve to surpass an arbitrarily set fluorescence intensity threshold serves as a direct measure of template concentration. Three no-template controls were prepared concurrently with each set of experimental samples and included in each amplification run to monitor any contamination generated during assay setup. Reactions were recorded and analyzed using the Applied Biosystems 7500 Fast System Sequence Detection System v2.0.3 (SDS v2.0.3).

2.5. SYBR Green (SG) qPCR assays

All SG qPCR assays used the Power SYBR Green PCR master mix (Applied Biosystems). The reactions were set up as described above in a total volume of 20 μL except that the Power SYBR Green PCR master mix was used and the TaqMan probe was omitted. Two microliters of each DNA sample was analyzed six times in the same manner as the TaqMan assays. The Power SYBR Green PCR master mix also included the dTTP/dUTP nucleotide mix and AmpErase UNG to protect against DNA contamination. Amplifications were performed under the same cycling conditions except for the post-amplification melting curve analysis (95 °C for 15 s, 60 °C for 1 min, a slow ramp of 1% to 95 °C for 15 s and 60 °C for 15 s). Three no-template controls were prepared to identify DNA contamination. The amplification reactions were recorded and analyzed using the ABI 7500 Fast System SDS v2.0.3. To determine the melting temperature of the amplicons generated during qPCR, the negative first derivative of the reporter fluorescence was plotted against the temperature using the ABI 7500 Fast System SDS v2.0.3 software.

2.6. Generation of standard curves

Accurate quantification of the Dhc biomarker gene requires that the absolute quantities of the gene copy are known. The Dhc sp. strain BAV1 16S rRNA gene inserted in the pcCR2.1 TOPO TA-cloning vector (Invitrogen) to generate pBAV1 was used as a template to generate standard curves. Gene copies were calculated as described (Ritalahti et al., 2006). Serial 10-fold dilutions of pBAV1 were carried out in triplicate from 3.38 × 10^8 plasmids to ~3.0 plasmids per μL and used as template DNAs in qPCR assays. By plotting the log of the calculated copy number against the cycle at which fluorescence for that sample crosses the threshold value, a standard curve was obtained. The Dhc target gene copy numbers of unknown samples were calculated automatically from the regression line by the ABI 7500 Fast System SDS v2.0.3 software. For comparison of data between different runs, standard curves were run with every plate and the data analyzed using automatic baseline settings and a set threshold value of 0.2 for all assays using both TaqMan and SG detection. To obtain the final result in copies per sample volume, calculated gene copies per qPCR reaction were divided by the template volume, multiplied by the total DNA elution volume, and the total divided by the sample volume (Ritalahti et al., 2006).

2.7. Agarose gel electrophoresis

PCR amplicons generated using the SG assay were separated by electrophoresis through 2.5% agarose Tris-acetate gels using 1× Tris-acetate-EDTA buffer (Sambrook and Russell, 2001). DNA bands were visualized after electrophoresis by staining with 0.5 μg ethidium bromide per mL buffer.

2.8. Statistical analysis

An analysis of covariance (ANCOVA) was used to determine if the slopes of the regression lines of the standard curves generated using the TaqMan and SG methods differed. The information from all dilution series was then combined using the unweighted Z-transformation approach (Whitlock, 2005) to obtain an overall p value for all the regression lines. Mean and standard deviations were determined for all gene abundance estimates using Microsoft Excel. A one-way analysis of variance was used to determine if gene abundance estimates differed between qPCR assays using TaqMan or SG detection chemistries. Statistical calculations were conducted using the JMP 8.0 software.

3. Results

3.1. Comparison of qPCR standard curves using both TaqMan and SG detection chemistries

Three independent, serial 10-fold dilutions of the pBAV1 plasmid served as templates in qPCR assays to generate standard curves using either the TaqMan or SG detection chemistry (Fig. 1). The standard curves generated for each dilution series resulted in regression lines with slopes that did not differ significantly from one another (ANCOVA Series 1, F_{1,44} = 1.484, p = 0.230, Series 2, F_{1,45} = 0.747, p = 0.382 and Series 3, F_{1,46} = 0.020, p = 0.889) (Fig. 1). When the data for the three series were combined and analyzed by unweighted Z-transformation (Whitlock, 2005), the slopes of the regression lines did not differ significantly from one another (Z = 0.122, p = 0.548). These data indicated that the TaqMan or SG detection chemistries did not affect the amplification efficiencies differently. In fact, the amplification efficiencies for using both types of qPCR assays were similar and ranged from 88.6 to 91.6% for the TaqMan and 89.7–90.9% for the SG assay. The y-intercepts indicated that the detection sensitivity ranged from 41.83 to 42.32 and 39.26–39.34 cycles for the TaqMan and SG assays, respectively. The y-intercept value corresponds to the Ct value for a single target molecule and values of ~40 indicate good assay sensitivity. The coefficients of correlation (R²), which serve as linearity indicators for the Ct values plotted in the standard curves, were greater than 0.997 and 0.992 for the TaqMan and the SG assays, respectively. The assays utilizing SG detection resulted in greater point scatter at <300 target gene copies per reaction compared with the results obtained with the TaqMan assay. The lowest template dilution of ~3 target gene copies per reaction produced non-specific amplification products thereby decreasing the sensitivity of the SG assay and resulting in a limit of detection (LOD) of 30 copies per reaction compared to a LOD of ~3 copies per reaction achieved with the TaqMan assay. The amplicons produced in the SG qPCR assays had melting temperatures (Tm) ranging from 77.0 to 78.9 °C.
which agrees with the theoretical $T_m$ of 77.1 °C for the expected 66-bp amplicon calculated with the OligoCalc v3.26 oligonucleotide properties calculator (Kibbe, 2007).

### 3.2. Quantification of Dhc 16S rRNA genes in defined laboratory cultures

Similar Dhc abundances were quantified using both TaqMan and SG detection in qPCR assays with template DNA obtained from Dhc pure cultures (e.g., strain BAV1) and dilutions of consortium KB-1. While some of the gene abundances were significantly different from one another using the two methods (Fig. 2), none of the target gene abundance estimates differed by greater than 3-fold (Fig. 2, BAV1-2, TaqMan 9.22 × 10^3 ± 1.85 × 10^0 gene copies/L, compared to SG 3.06 × 10^3 ± 2.49 × 10^0 gene copies/L) with most abundances differing by no more than 2-fold. The amplicons obtained with BAV1 pure culture DNA had $T_m$ values ranging from 77.4 to 79 °C and the amplicons generated with the KB-1 DNA ranged from 77.0 to 78.8 °C. In all cases, melting curve analysis exhibited a single, symmetric peak indicating a single $T_m$ value for the amplicons. Apparently, specific target gene amplification resulting in the formation of uniform amplicons was achieved with template DNA from the laboratory grown cultures.

### 3.3. Quantification of environmental samples using TaqMan and SG detection chemistries

With template DNA extracted from groundwater samples collected from nine wells at the Ft. Dix, NJ site, qPCR using the SG method generated quantifiable signals from all samples (Fig. 3). The abundance estimates for Dhc ranged from the highest abundance of 1.97 × 10^10 ± 1.42 × 10^6 gene copies/L for well BM-6 to the lowest abundance at 2.51 × 10^4 ± 1.86 × 10^4 gene copies/L for well EW-4. Initial analysis of the amplification in the samples BM-4, BM-8, EW-1, EW-2 and EW-4 (all samples with Dhc abundances <10^5 gene copies/L) suggested that PCR inhibitors were present in these samples (i.e., amplification curves for the 1:10 diluted template produced lower $C_T$ values compared to the undiluted template). The amplification curves produced for all the Ft. Dix samples were similar in shape to curves generated by amplification of the standard plasmid pBAV1. Amplification curves were also obtained from samples BM-2 and EW-4, in which TaqMan failed to detect the Dhc 16S rRNA gene target (Fig. 3). Surprisingly, the melting curve analysis of the BM-8 and EW-4 qPCR assays revealed multiple $T_m$ peaks and multiple temperatures were indicated by the ABI 7500 Fast software resulting in false-positive estimates for these samples.

In samples from wells BM-4 and EW-2, quantification using SG chemistry resulted in 2.7 and 2.9-fold higher Dhc 16S rRNA gene abundances for the same sample compared to those values determined using the TaqMan chemistry. For the BM-4 sample, melting curve analysis indicated that the samples using a 1:10 diluted template contained mostly nonspecific amplification products. In contrast, the melting curve analysis of duplicate undiluted template DNA samples for BM-4 suggested specific amplification with 6.85 × 10^3 ± 4.47 × 10^2 gene copies/L quantified, a 21-fold reduction in gene abundance compared to TaqMan-based enumeration of 1.46 × 10^5 ± 2.49 × 10^4 gene copies/L. The analysis of the EW-2 sample with the SG method yielded a single $T_m$ ranging from 77.2 to 77.7 °C for five of six replicates. This temperature matched the theoretical $T_m$ calculated using the expected amplicon sequence. The melting curves for the three replicate assays performed with the 1:10 diluted template DNA showed a single peak; however, the peak had a broad base uncharacteristic of uniform amplicons (Fig. 5E).

The remaining samples BM-2, BM-2x, BM-6 and EW-3 differed by not more than 4.8-fold (BM-2x, TaqMan 1.33 × 10^6 ± 2.41 × 10^5 gene copies/L compared to SG at 2.75 × 10^5 ± 1.22 × 10^5 gene copies/L) but the statistical analysis revealed significant differences between the results obtained with the TaqMan and the SG assay (One-way ANOVA, $p<0.05$ for EW-3 and $p<0.001$ for BM-2, BM-2x and BM-6) (Fig. 3). In each case, the TaqMan assay determined higher target gene abundances for the same sample compared to the SG assay, except for those cases described above.

### 3.4. Agarose gel electrophoresis of amplicons

In addition to melting curve analysis, the amplicons generated in the SG assay using template DNA from environmental samples were examined by agarose gel electrophoresis (Fig. 4). The formation of nonspecific amplicons was apparent in many of the samples assayed using template DNA derived from groundwater. In most cases, multiple additional bands or smears were observed indicating the presence of additional amplicons that were generally sized greater than 100 bp. Faint bands were observed in some no template control (NTC) reactions (Fig. 4, panel A, lane 6; panel B lane 6; panel D, lane...
affected by PCR inhibition as both undiluted and 1:10 diluted template PCR inhibition in assays with undiluted BMW-2 and BMW-2x dilution of the same DNA sample (data not shown) but gel electrophoresis revealed multiple amplicons (Fig. 4). The 1:10 diluted template DNA of sample EW-2 also produced a single Tm peak (ranging from 77.4 to 77.7 °C for three replicate assays) that matches the theoretical Tm of 77.1 °C for the expected amplicon sequence. Gel electrophoretic analysis revealed the formation of multiple nonspecific amplification products (Fig. 4D, lanes 2–4). The gel electrophoretic analysis also detected nonspecific amplicons in an undiluted EW-2 sample (Fig. 4C, lane 19). The combined melting curve and gel electrophoretic analyses revealed primer-dimer formation for another replicate of the EW-2 undiluted sample (Fig. 4C, lane 18), and resulted in the rejection of the qPCR results for both samples. Because five of six replicates for the EW-2 sample contained nonspecific amplicons, the SG assay failed to quantify Dhc abundance in sample EW-2. Furthermore, despite two of three replicates producing a single Tm peak in reactions using the undiluted BMW-4 sample (data not shown), the predominance of nonspecific amplification indicated by gel electrophoresis (Fig. 4, panel B, lanes 7–12), led to the rejection of all values generated for this sample, and resulted in a non-detectable (i.e., false-negative) score for the Dhc 16S rRNA gene using the SG chemistry. In contrast, the assay utilizing the TaqMan chemistry produced an abundance estimate of $1.46 \times 10^5 \pm 2.49 \times 10^4$ gene copies/L qPCR with template DNA from both BMW-8 and EW-4 samples yielded predominantly nonspecific amplicons, a result congruent with the melting curve analysis (data not shown) and resulting in a non-detect of the Dhc 16S rRNA gene in these samples.

3.5 Melting curve analysis artifacts

The formation of nonspecific amplicons in qPCR reactions that used template DNA from environmental samples was not always apparent from the traces of the melting curves (Fig. 5A–D) or from the Tm (Fig. 5). Only a single Tm peak at 78.4 °C that fell within the range of the standard curve melting temperatures was obtained for the 1:10 diluted FT. Dix BMW-6 sample (Fig. 5A). Yet, gel electrophoresis revealed two bands indicating nonspecific amplification for these samples (Fig. 5A, inset). Similarly, the melting curve analysis of qPCR reactions of Bachman site samples ML-3B and ML-3E indicated a single Tm that fell within the standard curve Tm range but gel electrophoresis revealed two amplicons (Fig. 5B and C). The melting curves for the Cecil field site 59 sample lw 31-2 indicated a single Tm of 80.1 and 80.3 °C for two replicates (Fig. 5D). In this instance, while the melting curves exhibited the same shape as the standard curve sample, the Tm recorded was just outside of the Tm range generated by the standard curve, and gel electrophoresis showed the presence of a second amplicon band (Fig. 5D, inset). A different phenomenon was observed in the analysis of the FT. Dix EW-2 and the Vandenbergh site 17B samples (Fig. 5E and F). Again, a single peak with one Tm was observed, although these samples produced multiple amplicons (i.e., multiple bands were observed in gel electrophoretic analysis). The shape of the peaks had a distinct broad base with shoulders, a characteristic that distinguished these melting curves from those with uniform amplicons (Fig. 5A–D).
4. Discussion

qPCR has emerged as the method of choice for enumeration of gene targets of interest, and information about Dhc biomarker gene abundances is used to support bioremediation at chlorinated solvent-contaminated sites. Different detection chemistries are used in qPCR analysis; however, their effects on the quality of the results are unclear. In this study, we compared abundance estimates of Dhc cells based on the quantification of the 16S rRNA gene and the use of the two most commonly utilized detection chemistries, the SG dye and a TaqMan probe. To minimize differences due to amplification efficiency of differently sized amplicons and/or different primer target sites, the evaluation used a single primer set for both chemistries in all qPCR assays. The abundance estimates of Dhc-containing consortia and pure cultures differed significantly between the two chemistries, but were within 3-fold of each other (Fig. 2) indicating that both methods generated comparable estimates of Dhc abundance when applied to pure cultures and consortia. A greater variability was seen with template DNA from environmental samples, and Dhc abundance estimates differed significantly ranging from a 2 to 21-fold difference between the two amplification detection chemistries. In reactions with template DNA from several environmental samples, the SG assays yielded false-positive results, overestimated Dhc abundance, and, in some cases, a predominance of nonspecific amplification obscured the specific signal (Fig. 4B). Additional analyses of the amplicons (i.e., melting curve and gel electrophoretic separation) ultimately produced a false-negative result for some samples due to exclusion of assay replicates that contained primer-dimers or nonspecific amplicons. The increased template specificity of the TaqMan approach avoided inflated Dhc biomarker gene abundance estimates and both false-positive and false-negative results.

The reasons why the TaqMan assays produced higher abundance estimates compared to the SG assays, except in those SG assays where nonspecific amplification contributed to the fluorescence signal, are unclear. Higher abundance estimates might be expected from SG detection compared to TaqMan because of higher specificity conferred in the TaqMan assay due to the requirement of probe binding. The only variables between the TaqMan and the SG assays were on gel insets). For the CEF59 Iw31-2 1:10 diluted sample, the expected amplicon is marked by a white arrowhead.

Fig. 5. Melting curve analysis of environmental samples assayed by SG qPCR. The negative first derivative of the normalized SG fluorescence was plotted against the temperature to determine the melting temperature (Tₘ) of the amplicons generated during qPCR analysis of six groundwater samples from four sites: Ft Dix, NJ; Bachman Site, ML; Cecil Field, Site 59, FL (CEF 59) and Vandenburg AFB, CA (VAN). Panels A, B, C and D indicate samples, in which the software identified only a single Tₘ, but two distinct amplicons are identified during agarose gel electrophoresis of the reaction products (samples corresponding to the traces are indicated on the agarose gel by solid black arrows). Panels E and F indicate samples, in which a single Tₘ was identified for each curve; however, agarose gel electrophoresis demonstrated both specific and multiple nonspecific amplification products. The Tₘ associated with sample melting curves are indicated. Amplicons produced by the standard curves were run with the samples where possible (marked as Std on gel insets).
the SG assay consistently yielded lower Dhc cell abundance estimates; however, the difference between both assays ranged from marginal to at most 4.8-fold lower numbers generated in the SG assays (some data depicted in Figs. 2 and 3).

Despite many methodological improvements for DNA extraction from environmental samples, effective recovery of DNA still remains a challenge, in particular from low biomass samples. Samples with low target DNA concentrations are problematic because such samples generally suffer from primer-dimer formation and mis-priming during PCR (Li et al., 1990). Primer-dimer formation has also been demonstrated to decrease PCR amplification efficiency (Rychlik, 1995). Both SG and TaqMan qPCR assays suffer from the formation of primer-dimers and mis-priming; however, the TaqMan probe discriminates between non-specific and specific amplicons and only the SG dye generates fluorescence from all double-stranded nucleic acids. The results of our study demonstrate that qPCR analysis using the SG dye can lead to erroneous quantification, in particular when applied to environmental samples with low template DNA concentrations. For example, the TaqMan assay quantified Dhc 16S rRNA genes in the EW-2 sample whereas the SG assay yielded an erroneous, false-negative result due to non-specific amplification identified by combined melting curve and gel electrophoresis analyses. The quantification of the BMW-4 sample using the SG assay yielded a false-negative result because the non-specific amplicons completely obscured the specific signal, whereas the TaqMan assay produced an abundance estimate.

Several reports have discussed the limitations and issues associated with the SG dye for quantitative analysis. Our study demonstrated reduced sensitivity with the SG chemistry due to non-specific amplification at low template concentrations, which resulted in a higher LOD compared to the TaqMan method. This limitation of the SG approach has been documented previously with a variety of targets including a human β-globin gene (Wittwer et al., 1997), a low concentration cytokine mRNA from human biopsies and rat tissues (Yin et al., 2001), and the thermonuclease gene of Staphylococcus aureus from contaminated cheeses (Hein et al., 2001). Another problem that occurs at high SG dye concentrations, is the dye's affinity to humic acids, which are often co-extracted with DNA from environmental samples, thereby affecting fluorescence measurements (Zipper et al., 2003). The SG dye also has greater affinity to AT-rich (Colborn et al., 2008; Zipper et al., 2004) and longer amplicons (Colborn et al., 2008; Giglio et al., 2003), which can bias target gene quantification. Yet another issue is the documented inhibitory effect of the SG dye on DNA amplification (Gudnason et al., 2007; Nath et al., 2000; Wittwer et al., 1997). Even at the low 2 μM SG dye concentration applied in the PCR assays, decreased amplification efficiencies have been reported (Gudnason et al., 2007). These confounding issues can affect the outcome of the analysis; however, the experimental design used in this study avoided uneven amplification in SG and TaqMan assays because both assay types used identical amplification primers and displayed similar amplification efficiencies as documented in Fig. 1. None of the issues impacting the SG method apply to the TaqMan assay (Colborn et al., 2008; Wittwer et al., 1997).

A distinct advantage usually ascribed to the SG dye is the opportunity to use melting curves for product differentiation during fluorescence monitoring of PCR (Ririe et al., 1997). While it has been argued that melting curve analysis can replace gel electrophoresis (Ririe et al., 1997), rigorous SG qPCR analysis requires both analytical procedures. The results with the groundwater samples demonstrated that the interpretation of melting curves is not always straightforward and can lead to erroneous conclusions. The Tm values calculated from the melting curves of the amplicons of the standard template DNA samples varied by up to 2 °C, complicating melting curve analysis for environmental samples (e.g., Cecil Field 59 lw 31-2 in Fig. 5D) that produced similarly shaped melting curves but differed by about 2 °C from the average standard curve Tm (78.5 °C). Gel electrophoretic analysis of sample lw 31-2 indicated the presence of both a specific and a nonspecific amplicon (Fig. 5D, inset). Similar observations were made in a previous study using a SG assay and primers targeting the Brucella abortus alkD gene and the neighboring IS711 insertion element. qPCR assay of 12 B. abortus strains yielded amplicons with multiple different Tm values (Newby et al., 2003). The same study demonstrated that bacterial species from genera such as Rhizobium, Sinorhizobium and Ochrobactrum that are closely related to the Brucella produced amplicons with Tm values similar to the B. abortus strains. The gel electrophoretic analysis of these amplicons indicated that only DNA templates from B. abortus strains yielded products of the expected size. In contrast, qPCR using the TaqMan chemistry did not generate signals from the closely related genera (Newby et al., 2003).

Despite a single Tm calculated for some melting curves, the shapes of the curves made it difficult to determine if the assay yielded specific amplicons (Fig. 5A–C, E and F). While obviously distorted melting curves indicated nonspecific product formation, slight distortions (Fig. 5A–C) or single peaks with broad bases (Fig. 5E and F) were difficult to interpret and gel electrophoresis was required to verify the presence/absence of heterogeneous amplicons (Fig. 5, insets). For example, the distorted melting curves obtained with the EW-2 diluted samples made the interpretation ambiguous and it was unclear whether these samples should be dismissed or used for calculating target gene abundances. Relying solely on Tm values reported by the instrument's software can be misleading and it is imperative that the shapes of the melting curves and the amplicons are analyzed. To complicate things further, melting curve analysis of a single amplicon type can yield more than a single peak in melting curve analysis. For example two peaks with distinct melting curve Tm values were obtained in qPCR targeting a region of the single stranded genome of the human circoviruses TT virus and TTV-like mini viruses, but gel electrophoretic and sequence analyses of the amplicons indicated homogeneous products (Moen et al., 2002). Taken together, our findings and previous reports indicate the necessity of post-qPCR analyses when the SG detection chemistry is used to ensure specific and accurate quantification and meaningful result interpretation. These findings indicate that the increased specificity achieved with the TaqMan detection chemistry provides advantages over the SG method, especially when the template DNA is derived from environmental samples. Despite the limitations noted for SG detection in qPCR assays, the SG approach has value when applied to templates obtained from defined samples, such as pure microbial cultures, and offers benefits for certain applications. If, for instance, many loci are targeted in qPCR analysis, it would be impractical to use the TaqMan method due to the financial expense and time involved in optimizing both primers and probes for each target.

Our findings have implications for the analysis of Dhc 16S rRNA genes in groundwater and the interpretation of the data. At contaminated sites where complete reductive dechlorination of chlorinated ethenes and ethene formation are observed, Dhc cell titers of greater than 10⁹ cells per liter are typically observed (Lu et al., 2006; Ritalahti et al., 2010a). At such high target gene concentrations, both the TaqMan and the SG methods would identify high Dhc cell titers and differences in the data obtained between the two methods would not lead to different conclusions regarding site management. A different scenario arises when Dhc are present at lower abundances and near the quantification limit, where the SG method can inflate the true numbers of Dhc cells or yield false-positive or false-negative results. For example, the erroneous conclusion that Dhc are present at a site may lead to the unproductive implementation of monitored natural attenuation (MNA) or biostimulation. Conversely, an inaccurate conclusion that Dhc are absent could lead to unnecessary bioaugmentation even though biostimulation might suffice. To draw meaningful conclusions, the data obtained with the SG method must be verified by additional analyses including melting curve and gel electrophoretic analyses.
While the focus of this study was on the Dhc 16S rRNA gene, the results apply for other gene targets as well. For researchers designing qPCR approaches for abundance estimates of genes of interest in environmental samples, the outcome of this research indicates advantages of the TaqMan approach and recommends the following strategy: (i) design primers for the TaqMan method, (ii) optimize the primers and the PCR conditions using the SG detection chemistry (i.e., eliminate or minimize mis-priming and/or primer-dimer formation), and (iii) use the optimized PCR conditions with the TaqMan probe. For researchers relying on other (e.g., commercial) laboratories for qPCR analysis, it is imperative that appropriate quality control is carried out independent of the method used. While qPCR has become a standard technique for quantification of nucleic acid biomarkers, there is a trend for omitting relevant technical information in scientific reports (Bustin et al., 2009; Cupples, 2008).

In fact, many studies that use the SG detection chemistry fail to mention whether amplicons were analyzed by melting curve analysis and/or gel electrophoresis. Our findings emphasize the need for these additional analyses when the SG assay is used, in particular when it is applied to environmental samples. These additional steps are required to establish confidence in the qPCR data obtained with the SG method, in particular if the data are used to support contaminated site management decisions (e.g., the implementation of bioremediation).

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References


