Sulphate-reducing bacteria (SRB) play a significant role in the mineralisation of organic matter in anaerobic environments and in the biogeochemical cycling of sulphur. In environments rich in sulphate, sulphate reduction dominates mineralization, accounting for up to 50% of the organic matter decomposition in estuarine and coastal marine sediments (Jørgensen 1982). To enumerate SRB from natural environments, traditional culture-dependent methods have previously been employed. Several studies, however, have demonstrated the numbers of viable SRB in aquatic sediments are considerably underestimated when standard most-probable-number (MPN) methods are used with selective enrichment media (Gibson et al. 1987, Jørgensen 1978).

Dissimilatory sulphite reductase (DSR) is the primary enzyme in the dissimilatory sulphate reduction in sulphate-reducing prokaryotes. DSR catalyses the six-electron reduction of (bi)sulphite to sulphide. This is the final step in sulphate respiration, a reaction found only in dissimilatory sulphate-reducing prokaryotes. The ubiquity of DSR in all known sulphate-reducing prokaryotes and its highly conserved nucleotide sequence make this enzyme ideal for assessing the diversity of sulphate-reducing prokaryotes in nature (Wagner et al. 1998, Zverlov et al. 2005). Using new assays for the polymerase chain reaction (PCR) amplification of fragments from the gene coding for the α-subunit (dsrA) and β-subunit (dsrB) of DSR, many laboratories are studying the diversity and distribution of SRB in aquatic environments (Chang et al. 2001, Joulian et al. 2001, Pérez-Jiménez et al. 2001, Thomsen et al. 2001). We previously developed new PCR primers selective for the dsrA of most mesophilic SRB belonging to β-Proteobacteria and used a quantitative competitive PCR for sensitive detection and enumeration of SRB populations as an alternative to culture-dependent methods (Kondo et al. 2004). This primer set has been used widely for detecting and enumerating SRB from marine and limnic environments (Kawahara et al. 2008, Kondo et al. 2006, 2007, Kondo & Butani 2007, Leloup et al. 2007, Neretin et al. 2007, Schippers & Neretin 2006, Webster et al. 2006). Schippers & Neretin (2006) and Leloup et al. (2007) have already used quantitative real-time PCR (qPCR) using our primer set for enumerating SRB in marine sediments, but there is no report on the application for water samples. Here we describe a method for rapid enumeration of SRB from aquatic environments using a quantitative real-time PCR (qPCR) in combination with a rapid DNA isolation method. Enumeration of SRB in the sediment and water samples was performed by quantifying the copy number of the dsrA gene coding for the α-subunit of the dissimilatory sulphite reductase using real-time PCR with the SYBR Green I assay. Using dsrA-specific primers, we demonstrated that quantification of SRB in known numbers of SRB assemblages can be achieved. We compared DNA isolation methods using commercial DNA extraction kits and a published technique. We found two commercial kits were of advantage for extraction of DNA from water or sediment samples, where a large number of samples could be processed at the same time. We showed this newly developed qPCR technique targeting dsrA is rapid, simple and reproducible for the quantification of SRB numbers in situ and is superior to the use of culture-dependent techniques.

**Key words:** dissimilatory sulphite reductase, DNA extraction, real-time PCR

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**Abstract:** We describe a rapid and simple enumeration method for sulphate-reducing bacteria (SRB) from aquatic environments using a quantitative real-time PCR (qPCR) in combination with a rapid DNA isolation method. Enumeration of SRB in the sediment and water samples was performed by quantifying the copy number of the dsrA gene coding for the α-subunit of the dissimilatory sulphite reductase using real-time PCR with the SYBR Green I assay. Using dsrA-specific primers, we demonstrated that quantification of SRB in known numbers of SRB assemblages can be achieved. We compared DNA isolation methods using commercial DNA extraction kits and a published technique. We found two commercial kits were of advantage for extraction of DNA from water or sediment samples, where a large number of samples could be processed at the same time. We showed this newly developed qPCR technique targeting dsrA is rapid, simple and reproducible for the quantification of SRB numbers in situ and is superior to the use of culture-dependent methods.

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the dsrA specific primers for mesophilic SRB described elsewhere (Kondo et al. 2004, Leloup et al. 2007): DSR-1F (+3'-ACSCACTGGAAGCAGCGCCG-3') and DSR-R (5'-GTG-GMRCCGTCGCAKRTTG-3'). The reaction mixture (50 μL) was: 25 μL of Master Mix (2x QuantiTect® SYBR® Green, Qiagen), 2 μL of 25 mM MgCl₂, 10 μL of 5x Q-solution (if required), 1 μL each of 20 μM primer and 1 μL of template DNA extract. qPCR was performed using an iCycler iQ system (Bio-Rad) as follows: 15 min at 95°C for initial denaturation; and 40 cycles: 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C.

We previously used 3.5 mM MgCl₂ in the Q-solution of the PCR mixture to detect the dsrA gene from aquatic environments (Kawahara et al. 2008, Kondo et al. 2004, 2006, 2007, Kondo & Butani 2007). We determined the effects of MgCl₂ concentrations to establish optimal qPCR assay conditions. The plasmid pCRII (Invitrogen) containing an approximately 1.9 kbp-length of the dsrAB from Desulfovibrio desulfuricans DSM642² was constructed with the dsr-1F and dsr-4R primers (Wagner et al. 1998); and was used as the standard for the calibration curve. As shown in Fig. 1, the threshold cycle (Ct) value decreased linearly with the initial concentration of dsrA gene in the PCR mixture. PCR efficiency using 3.5 mM MgCl₂ without Q-solution was 93.5%, which was higher than that using 2.5 mM MgCl₂ (90.1%). The PCR efficiency with the Q-solution was 65.5% or very low compared to not using the Q-solution. Q-solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions (Taq PCR Handbook, Qiagen). The Q-solution caused reduced efficiency in the qPCR system. This is possibly due to differences in PCR chemistry between standard Taq PCR and QuantiTect® SYBR® Green PCR systems. We used 3.5 mM MgCl₂ without the Q-solution in our standard qPCR mixture. The Ct value decreased linearly with amount between 10² and 10¹⁰ copies of dsrA gene in the PCR mixtures.

The qPCR amplification of dsrA using our standard qPCR conditions was tested using different concentrations of the plasmid pCRII containing dsrAB genes from the following SRB species as the templates: Desulfovibrio desulfuricans DSM642², Desulfobacterium autotrophicum DSM3382², Desulfolubus propionicus DSM2032², Desulfobacter latus ATCC43918² and Desulfococcus multivorans DSM2059². All of the templates gave reliable exponential fluorogenic amplification patterns dependent on the initial template amount in the range from ~10² to 10¹⁰ copies of the dsrA genes (Fig. 2). This shows that the qPCR assay can quantify a wide range of dsrA copy numbers compared to the competitive PCR technique as described previously (Kondo et al. 2004).

Thereafter, we examined various DNA isolation methods for sediment and water samples to enumerate SRB that may be easily applied to a large number of samples. Desulfovibrio desulfuricans DSM642² was used in the calibration of water and sediment samples. The strain was grown in Postgate’s C medium (Postgate 1984) and the bacterial cells were counted using the 4,6-diamidino-2-phenylindole (DAPI) method (Porter & Feig 1980). The cells were centrifuged at 14,400 g for 20 min at 4°C; resuspended to about 10¹¹ cells mL⁻¹; and serially diluted. For the water samples, the cells were collected on sterile polycarbonate membrane filters (0.2 μm, Advantec). The filters were transferred into 2 mL screw-cap tubes containing 0.5 g of baked glass beads for the hydroxyapatite (HTP) spin-column method (Purdy et al. 1996) or Lysing Matrix tubes of the FastDNA SPIN Kit (MP Biomedicals, LLC) or the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC). DNA isolations were performed for each serially diluted sample using the HTP spin-column method (Purdy et al. 1996), the FastDNA SPIN Kit or the FastDNA SPIN Kit for Soil. DNAs were suspended in 50 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) or DNase/Pyrogen free water. The copy number of the dsrA gene in each of the samples was measured under the optimal qPCR conditions, and the data showed a lin-

![Fig. 1. Standard curves for the dsrA gene from Desulfovibrio desulfuricans DSM642². The threshold cycle values are plotted against the input dsrA gene copy number. The differences in the slope and y-intercept for the dsrA gene standard curves amplified without Q-Solution (○) for 3.5 mM MgCl₂ and ● for 2.5 mM MgCl₂) compared with the dsrA gene standard curve amplified with Q-Solution (▲) indicate a difference in amplification efficiency and sensitivity, respectively.](image1)

![Fig. 2. PCR product detection of dsrA from various SRB strains using real-time PCR. Representative dsrA genes were obtained from Desulfovibrio desulfuricans DSM642² (▲), Desulfobacterium autotrophicum DSM3382² (●), Desulfolubus propionicus DSM2032² (○), Desulfobacter latus ATCC43918² (▲) and Desulfococcus multivorans DSM2059² (●).](image2)
ear relationship ($r > 0.99$) between the cell numbers collected on filters and those detected by qPCR when the HTP spin-column method and the FastDNA SPIN Kit were used (Fig. 3). However, the FastDNA SPIN Kit for Soil was found to give only low efficiency for DNA extraction from filtered samples. The slopes of the calibration curves were found to be 1.02 for the HTP spin-column method and 1.09 for the FastDNA SPIN Kit showing the assays slightly overestimate the number of *Desulfovibrio desulfuricans* cells that were filtered compared to DAPI counting. The data shows the qPCR of DNA from $10^3$ to $10^{10}$ cells can be used for quantification in the filtered samples. Although there was no difference in counts of SRB from filtered samples using qPCR between the HTP spin-column and the FastDNA SPIN Kit isolation, the FastDNA SPIN Kit has the advantage that a large number of samples can be processed in a shorter (at least less than half) period of time compared to the HTP spin-column method. The reproducibility of the measurements using the FastDNA SPIN Kit was determined using a water sample collected from 20 m-depth located in the central basin of the meromictic Lake Suigetsu (35°35'N, 135°53'E). The mean value of eight replicate measurements by the qPCR was $8.0 \times 10^6$ copies mL$^{-1}$ of water with a standard deviation of $2.3 \times 10^2$ copies mL$^{-1}$. The coefficient of variation was 28%. The reproducibility of the measurements of *dsrA* copy number was lower than that gained using competitive PCR (Kondo et al. 2004), but was superior to the culture-dependent method (MPN counting).

To determine if SRB could be detected using qPCR with a background of nonspecific DNA from sediment, sediment samples were collected at the same station as for the water samples, and were spiked with known numbers of *D. desulfuricans* DSM642$^T$ cells. A 0.5-g sediment aliquot was transferred into 2 mL screw-cap tubes containing 0.5 g of baked glass beads for the HTP spin-column method or Lysing Matrix tubes of the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC). DNA isolation was performed with the spiked samples using the HTP spin-column method or FastDNA SPIN Kit for Soil. DNA from each of the samples was quantified using the optimal qPCR conditions. The data shows a linear relationship between the number of cells added in the spike and the number of cells detected (Fig. 4). Using each of the DNA isolation methods, the calibration curves were similar. In common with the filtered samples, the FastDNA SPIN Kit for Soil can be used with a larger number of sediment samples than the HTP spin-column method. The reproducibility of the measurements with the FastDNA SPIN Kit for Soil was determined using the unspiked sediment sample. The mean value of ten replicate measurements using qPCR was $5.3 \times 10^6$ copies g$^{-1}$ wet sediment with a standard deviation of $1.2 \times 10^4$ copies g$^{-1}$. The coefficient of variation was 22%. Schippers & Neretin (2006) and Leloup et al. (2007) have used the FastDNA SPIN Kit for Soil for isolation of DNA from marine sediments, while an additional purification of DNA was needed for the application of *dsrA* quantification by qPCR. In our case, the standard FastDNA SPIN Kit or FastDNA SPIN Kit for Soil protocol was sufficient to quantify *dsrA* from aquatic environments.

A comparison of quantification of SRB between competitive PCR and qPCR methods has been carried out using the same DNA samples as used in the previous report on SRB distribution in fish farm sediments (Kawahara et al. 2008) and in the water column of a meromictic lake (Kondo & Butani 2007). DNA from each of the samples was amplified under the optimal competitive PCR or qPCR conditions and the results show a linear relationship ($r=0.995$) between the SRB cell densities determined by the two methods (Fig. 5). There were slight differences in the numbers of SRB compared to the ideal ($y=x$), which is probably due to experimental factors. This result indicates that the qPCR assay for SRB enumeration gives nearly identical results to competitive PCR.

![Fig. 3.](image1.png) Calibration curves of SRB counts using real-time PCR for filtered samples. DNA was isolated using the HTP spin-column method (●), the FastDNA SPIN Kit (○) and the FastDNA SPIN Kit for Soil (▲). Error bars indicate the standard error of the mean (n=3).

![Fig. 4.](image2.png) Calibration curves showing the *dsrA* gene copy numbers determined by real-time PCR for sediment samples with the addition of different numbers of *Desulfovibrio desulfuricans* DSM642$^T$ cells before DNA extraction. DNA was isolated using the HTP spin-column method (●) and the FastDNA SPIN Kit for Soil (○). Error bars indicate the standard error of the mean (n=3).
Density of SRB determined by competitive PCR (log cells g⁻¹ dry sediment or mL⁻¹ water)

Fig. 5. Relationship between sulphate-reducing bacterial (SRB) densities in fish farm sediments (filled symbols) and water columns of a meromictic lake (open symbols) determined by competitive PCR and qPCR methods. Dotted line denotes a regression equation derived from all the data used in the comparison. Error bars indicate the standard error of the mean (n=3).

We found qPCR with the commercial DNA isolation kit to be easy, accurate and reliable for SRB quantification. Determination of the dsrA gene or SRB cells from sediment and water samples using DNA isolation and qPCR can be performed within a day. Our data suggest this is a rapid, sensitive and reproducible quantification of SRB population sizes in aquatic environments and is a superior alternative to the use of culture-dependent techniques.

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