

## Development and Application of a Real-Time PCR Approach for Quantification of Uncultured Bacteria in the Central Baltic Sea

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We have developed a highly sensitive approach to assess the abundance of uncultured bacteria in water samples from the central Baltic Sea by using a noncultured member of the “*Epsilonproteobacteria*” related to *Thiomicrospira denitrificans* as an example. Environmental seawater samples and samples enriched for the target taxon provided a unique opportunity to test the approach over a broad range of abundances. The approach is based on a combination of taxon- and domain-specific real-time PCR measurements determining the relative *T. denitrificans*-like 16S rRNA gene and 16S rRNA abundances, as well as the determination of total cell counts and environmental RNA content. It allowed quantification of *T. denitrificans*-like 16S rRNA molecules or 16S rRNA genes as well as calculation of the number of ribosomes per *T. denitrificans*-like cell. Every real-time measurement and its specific primer system were calibrated using environmental nucleic acids obtained from the original habitat for external standardization. These standards, as well as the respective samples to be measured, were prepared from the same DNA or RNA extract. Enrichment samples could be analyzed directly, whereas environmental templates had to be preamplified with general bacterial primers before quantification. Preamplification increased the sensitivity of the assay by more than 4 orders of magnitude. Quantification of enrichments with or without a preamplification step yielded comparable results. *T. denitrificans*-like 16S rRNA molecules ranged from  $7.1 \times 10^3$  to  $4.4 \times 10^9$  copies ml<sup>-1</sup> or 0.002 to 49.7% relative abundance. *T. denitrificans*-like 16S rRNA genes ranged from  $9.0 \times 10^1$  to  $2.2 \times 10^6$  copies ml<sup>-1</sup> or 0.01 to 49.7% relative abundance. Detection limits of this real-time-PCR approach were 20 16S rRNA molecules or 0.2 16S rRNA gene ml<sup>-1</sup>. The number of ribosomes per *T. denitrificans*-like cell was estimated to range from 20 to 200 in seawater and reached up to 2,000 in the enrichments. The results indicate that our real-time PCR approach can be used to determine cellular and relative abundances of uncultured marine bacterial taxa and to provide information about their levels of activity in their natural environment.

Many of the molecular techniques currently used in microbial ecology lack a quantitative component. Nevertheless, quantification of microbial abundances is of great relevance for the understanding of microbial community structure and dynamics in natural environments. Certainly, for enumeration and spatial localization of specific microorganisms in natural samples, whole-cell fluorescence in situ hybridization techniques hold considerable promise (14). However, PCR-based techniques provide an alternative that could be more sensitive and less labor intensive. For example, this has been demonstrated for the ultramicrobacterium *Sphingomonas alaskensis*, which could not be detected by fluorescence in situ hybridization, despite the ability to detect it by PCR (5, 33).

Real-time PCR is a new and highly sensitive method for the quantification of microorganisms from different samples (40). Typical quantitative PCR (Q-PCR) techniques utilize approaches originally developed for clinical applications. Q-PCR has been used for the quantification of taxa and target genes from various bacteria, such as *Staphylococcus aureus* (15), *Ba-*

*cillus anthracis* (24, 30), and *Legionella* spp. (45), and from viruses (11) or for the quantification of insulin-like growth factors (28). Recently, applications of real-time PCR have also been demonstrated with marine samples to quantify diatoms and pelagophytes (41) and numbers of cells of *Synechococcus* spp., *Prochlorococcus* spp. (37), and *Pseudomonas stutzeri* (12); with soil samples to quantify cells of *Rhodococcus* spp. (31), methanotrophs (19), and fungi (9); with sediments to quantify cells of *Geobacter* spp. (36); in wastewater treatment plants to quantify nitrifying bacteria (13); and with cultures to determine dissimilatory (bi)sulfite reductase gene expression in *Desulfobacterium autotrophicum* (26). One advantage of PCR techniques, apart from their great potential for automation, is their sensitivity. Theoretically, PCR should detect one copy of the 16S rRNA gene (39). Lockey et al. (21) described a real-time PCR method and validation strategy useful to detect a single-copy herpes simplex virus thymidine kinase gene. Moreover, a single *B. anthracis* spore was detected in 100 liters of air sampled by real-time PCR (24). However, for the most part, sensitivity becomes limited below 100 gene copies per reaction mixture (12, 35).

Sensitivity and specificity can be improved by adopting a “nested” or a “heminested” PCR approach, whereby initial amplification is carried out with a pair of primers with broad specificity (14, 38). This has been successfully used to detect

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TABLE 1. Comparison of total 16S rRNA contents from seawater samples and enrichments<sup>a</sup>

Sample	Depth (m) or source of enrichment (sample)	Total 16S rRNA content (ng/ml)	Total 16S rRNA (no. of molecules/ml) <sup>b</sup>	<i>T. denitrificans</i> -like-organism-specific 16S rRNA fraction		Total cells (10 <sup>6</sup> /ml) <sup>c</sup>	<i>T. denitrificans</i> -like-organism-specific 16S rRNA gene fraction (%) relative abundance)	No. of <i>T. denitrificans</i> -like cells/ml	No. of ribosomes/ <i>T. denitrificans</i> -like cell
				% Relative abundance	No. of molecules/ml				
G1	5	1.41 (±0.04)	1.62 × 10 <sup>9</sup> (±4.48 × 10 <sup>7</sup> )	0.003 (±0.003)	4.86 × 10 <sup>4</sup> (±1.34 × 10 <sup>3</sup> )	2.15	0.012 (±0.002)	2.58 × 10 <sup>2</sup> (±4.30 × 10 <sup>1</sup> )	200
G2	30	0.06 (±0.02)	6.59 × 10 <sup>7</sup> (±2.09 × 10 <sup>7</sup> )	0.059 (±0.018)	3.89 × 10 <sup>4</sup> (±1.23 × 10 <sup>4</sup> )	1.31	—	—	—
G3	50	0.31 (±0.01)	3.54 × 10 <sup>8</sup> (±1.46 × 10 <sup>7</sup> )	0.002 (±0.001)	7.10 × 10 <sup>5</sup> (±2.92 × 10 <sup>2</sup> )	0.53	—	—	—
G4	80	0.21 (±0.00)	2.44 × 10 <sup>8</sup> (±9.51 × 10 <sup>5</sup> )	0.0036 (±0.0024)	8.78 × 10 <sup>3</sup> (±3.42 × 10 <sup>1</sup> )	0.43	0.021 (±0.007)	9.00 × 10 <sup>1</sup> (±3.01 × 10 <sup>1</sup> )	100
G6	120	0.29 (±0.01)	3.32 × 10 <sup>8</sup> (±6.43 × 10 <sup>6</sup> )	0.083 (±0.038)	2.76 × 10 <sup>6</sup> (±5.34 × 10 <sup>4</sup> )	0.27	1.552 (±0.158)	4.19 × 10 <sup>3</sup> (±4.27 × 10 <sup>2</sup> )	70
G7	138	0.46 (±0.01)	5.24 × 10 <sup>8</sup> (±8.75 × 10 <sup>6</sup> )	0.916 (±0.251)	4.80 × 10 <sup>6</sup> (±8.02 × 10 <sup>4</sup> )	0.85	8.346 (±3.110)	7.08 × 10 <sup>4</sup> (±2.64 × 10 <sup>4</sup> )	70
G9	175	0.48 (±0.02)	5.53 × 10 <sup>8</sup> (±1.97 × 10 <sup>7</sup> )	0.466 (±0.075)	2.58 × 10 <sup>6</sup> (±9.18 × 10 <sup>4</sup> )	0.90	15.055 (±3.519)	1.35 × 10 <sup>5</sup> (±3.17 × 10 <sup>4</sup> )	20
G10	225	0.04 (±0.00)	4.65 × 10 <sup>7</sup> (±3.78 × 10 <sup>6</sup> )	—	—	1.10	—	—	—
Bx21	G7	3.27 (±0.22)	3.76 × 10 <sup>9</sup> (±2.63 × 10 <sup>8</sup> )	8.445 (±0.826)	3.18 × 10 <sup>8</sup> (±3.11 × 10 <sup>7</sup> )	1.66	17.850 (±2.709)	2.96 × 10 <sup>5</sup> (±4.50 × 10 <sup>4</sup> )	1,100 <sup>d</sup>
Bx23	G9	3.23 (±0.13)	3.71 × 10 <sup>9</sup> (±1.45 × 10 <sup>8</sup> )	19.099 (±1.070)	7.09 × 10 <sup>8</sup> (±3.97 × 10 <sup>7</sup> )	1.31	38.894 (±9.100)	5.10 × 10 <sup>5</sup> (±1.19 × 10 <sup>5</sup> )	1,400 <sup>e</sup>
Bx24	G9	7.74 (±0.10)	8.90 × 10 <sup>9</sup> (±1.19 × 10 <sup>9</sup> )	49.723 (±9.951)	4.43 × 10 <sup>9</sup> (±8.86 × 10 <sup>8</sup> )	4.43	49.654 (±8.001)	2.20 × 10 <sup>6</sup> (±3.54 × 10 <sup>5</sup> )	2,000 <sup>f</sup>

<sup>a</sup> Total 16S rRNA contents from seawater samples and enrichments with *T. denitrificans*-like-organism-specific relative and cellular 16S rRNA and 16S rRNA gene abundances and estimated number of ribosomes per *T. denitrificans*-like cell. Standard deviations are given in parentheses. —, below detection limit.

<sup>b</sup> Based on 1.15 × 10<sup>9</sup> molecules of 16S rRNA/ng.

<sup>c</sup> Data from Weinbauer et al. (44).

<sup>d</sup> Value indicates a 16-fold increase of ribosome content per cell in comparison to that of the original environmental sample.

<sup>e</sup> Value indicates a 70-fold increase of ribosome content per cell in comparison to that of the original environmental sample.

<sup>f</sup> Value indicates a 100-fold increase of ribosome content per cell in comparison to that of the original environmental sample.

autotrophic ammonia-oxidizing bacteria in samples of lake water (14) or in combination with species-specific PCR product melting temperatures (with SybrGreen) to identify pathogenic *Campylobacter* species (23).

Our main interest is the ecology of microbial catalysts involved in major biogeochemical processes of the central Baltic Sea. Previous studies (3, 4) have shown that autotrophic denitrification may be an important process for N balance in the central Baltic Sea. Thus, in the present study, bacteria responsible for autotrophic denitrification were chosen as a target for our research. In a combined experimental and molecular approach, a bacterium related to *Thiomicrospira denitrificans* was identified as a major catalyst. Quantification of bacterial catalysts independent from their ribosome contents or the necessity of culturing these bacteria is a tool urgently needed for elucidating their ecology. We developed a specific Q-PCR technique allowing quantification of *T. denitrificans*-like bacteria both in experimental enrichments and in environmental samples. The method is based on specific primers (OST 1R and OST 1F) designed from a consensus sequence obtained from community fingerprints of Baltic bacterioplankton (M. G. Höfle, S. Flavier, R. Christen, J. Bötel, M. Labrenz, and I. Brettar, submitted for publication). The method gave reliable results when it was applied to DNA as well as to RNA extracted from bacterioplankton. Our approach allowed the in situ detection of a bacterial catalyst under consideration down to 90 cells ml<sup>-1</sup> or a relative abundance of 0.01% without the need of absolute standards in real-time experiments. Based on total bacterial cell counts, the number of ribosomes per cell could also be estimated. This approach may help to improve quantification of major bacterial catalysts, especially for oligotrophic environments, and allow us to gain new insights into their in situ ecology.

## MATERIALS AND METHODS

**Sampling, analysis of samples, and growth conditions.** Water samples were taken at depths of 5 to 225 m in the central Baltic Sea (Gotland Deep) in September 1998 (Table 1). Sample handling and physicochemical analysis have been described elsewhere (3, 10). The samples were filtered immediately onto a sandwich of a Nucleopore filter (pore size, 0.2 μm) and a glass fiber filter (GF/F; Whatman) and stored frozen for later analysis (16). To enrich autotrophic denitrifiers, water samples from the oxic-anoxic interface at 138 m (in situ concentrations: NO<sub>3</sub>, 0.4 μmol liter<sup>-1</sup>; H<sub>2</sub>S, 5.7 μmol liter<sup>-1</sup>; no O<sub>2</sub>), above the interface at 120 m (O<sub>2</sub>, 0.3 ml; NO<sub>3</sub>, 8.0 μmol liter<sup>-1</sup>; no H<sub>2</sub>S), and below the interface at 175 m (H<sub>2</sub>S, 24 μmol liter<sup>-1</sup>; no NO<sub>3</sub>) were incubated at 5°C in the dark in gastight 1.2-liter bottles after the addition of 150 μmol of KNO<sub>3</sub> liter<sup>-1</sup> and 100 μmol of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> liter<sup>-1</sup>. Controls were run without substrate addition. After 44 h (samples Bx21 and Bx23) and 88 h (sample Bx24), enriched water samples were processed analogously to the untreated water samples. Total bacterial counts were determined as described by Weinbauer et al. (44).

**Nucleic acid extraction and quantification.** To process the nucleic acids of the water samples harvested on sandwich filters, nucleic acid extraction and quantification from the frozen filters were performed by parallel extraction of RNA and DNA (using a phenol extraction protocol) as described by Weinbauer et al. (43). Nucleic acid concentrations were determined fluorometrically (42). Prior to RT-PCR and fluorometric analysis, RNA extracts were treated with DNase I (Roche Diagnostics, Mannheim, Germany) for 60 min at 37°C.

**Primers.** The application of 16S ribosomal complementary DNA (rcDNA) single-strand conformation polymorphism analysis and sequencing of dominant bands (29) demonstrated that an autotrophic denitrifying bacterium related to, but not identical with, *T. denitrificans* was the dominant autotrophic denitrifier in the above-described enrichments (Höfle et al., submitted). Subsequent development of highly specific primers against this bacterium has also been described in detail (Höfle et al., submitted). *T. denitrificans*-like forward primer OST 1F (5'-TCAGATGTGAAATCCAATGGCTCA-3') corresponds to 16S rRNA

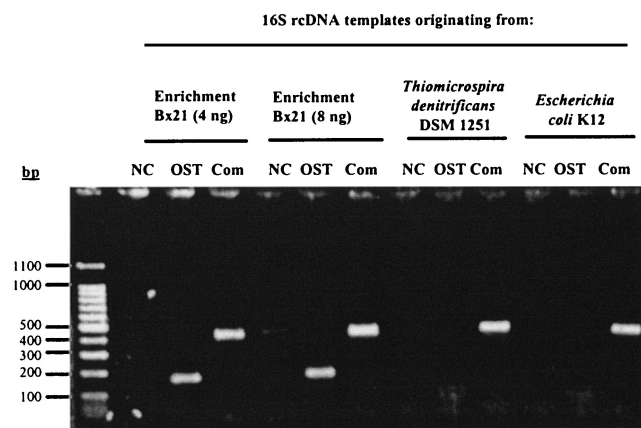


FIG. 1. Agarose (1.2%) gel of RT-PCR amplification products based on the OST 1F-OST 1R (OST) or Com 1-Com 2ph (Com) primer system from various template RNAs. Templates originated from the thiosulfate-plus-nitrate enrichment Bx21, *T. denitrificans* DSM 1251<sup>T</sup>, or *E. coli* K-12. The first lane contains molecular size markers (100 bp). NC, negative control.

*Escherichia coli* positions 663 to 686, and reverse primer OST 1R (5'-CTTAGC GTCAGTTATGTCCAGG-3') corresponds to *E. coli* positions 803 to 825. Primers used for bacterial 16S rRNA gene and 16S rDNA amplification from environmental DNA or RNA are given by Schwieger and Tebbe (34). Primer set Com 1 (5'-CAGCAGCCGCGTAATAC-3') and phosphorylated Com 2ph (5'-CCGTC AATTCCTT GAGTTT-3') amplify positions 519 to 926 of the *E. coli* 16S rRNA gene.

**16S rDNA generation.** To retrieve 16S rDNA, 30 ng of template RNA was reverse transcribed at 50°C with the ThermoScript reverse-transcription (RT)-PCR system (Invitrogen) and the universal reverse primer 1492R (5'-GGTTA CCTTGT TACGACTT-3') (20), according to the manufacturer's instructions. In each reverse transcription reaction mixture, some RNA samples were not supplemented with reverse transcriptase to rule out DNA contamination.

**Relative quantification protocol without a preamplification step.** A Rotor-Gene real-time cycler (Corbett Research), the Brilliant SybrGreen Q-PCR master mix (Stratagene), and nucleic acids isolated from Baltic seawater experiment (Bx) enrichments most likely containing large amounts of the target species were used for the establishment of Q-PCR and quantitative RT-PCR (Q-RT-PCR) protocols. Prior quantifications, minimum Com and OST primer concentrations (in a range from 50 to 900 nM for each primer), and optimal template concentrations were determined. For optimal primer binding, a temperature of 59°C was determined, where no binding to the sequence of the most closely related bacterium which could be cultured, i.e., *T. denitrificans*, occurred (Fig. 1). The optimized PCR mixtures (25  $\mu$ l) contained 1 $\times$  Brilliant SybrGreen Q-PCR master mix (Stratagene), the Com (a 900 nM concentration of each primer) or OST 1 (OST 1F, 900 nM; OST 1R, 300 nM) primer system, and 2 to 4 ng of nucleic acids extracted from the Bx enrichments. An initial denaturing step at 95°C for 15 min was followed by 40 cycles of 94°C for 30 s, 59°C for 40 s, and 72°C (a measuring step) for 50 s. Potential development of primer dimers was determined by a melting-point analysis in a range from 40 to 94°C. Each measurement was performed multiple times (at least three replicates).

**Relative quantification protocol including a preamplification step.** Four nanograms of generated 16S rDNA or 2 to 4 ng of chromosomal DNA was preamplified according to the method of Teo et al. (38), using the universal primer 1492R and the *Bacteria*-specific forward primer 27F (5'-AGAGTTTGATCCTG GCTCAG-3') (20). PCR mixtures contained 1 $\times$  PCR buffer, a 200  $\mu$ M concentration of each deoxynucleoside triphosphate, 150 nM concentrations of the forward and reverse primers, and 0.025 U of Hot Star Taq (Qiagen)/ $\mu$ l. An iCycler (Bio-Rad) was used to incubate reaction mixtures through an initial denaturation at 95°C for 15 min, followed by 16 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Additional Q-PCR and Q-RT-PCR protocols are as described above. In order to check the comparability of the two protocols, Bx enrichments were quantified with and without preamplification steps.

**Real-time data analysis.** Relative standards were prepared using serially diluted (1:10) nucleic acids isolated from exactly the same DNA or RNA extract. Each sample measured was calibrated by its own respective Com and OST

standards. Due to potentially lower PCR efficiencies of nondiluted DNA or cDNA extracts, each sample to be measured was always diluted 1:10. Calibration curves were generated by Rotor-Gene software version 4.6. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or cycle threshold ( $C_T$  value). The slope of each calibration curve was incorporated into the following equation to determine the reaction efficiency: efficiency =  $10^{(-1/\text{slope})} - 1$ . According to this formula, an efficiency of 1 means a doubling of product in each cycle. Only data resulting from measurements with comparable Com- and OST-specific PCR efficiencies were used for further calculations.

**Calculation of numbers of *T. denitrificans*-like cells and 16S rRNA molecules.** In order to calculate numbers of *T. denitrificans*-like cells, total cell numbers were used and the Com product/OST product ratios were obtained from relative 16S rRNA gene quantifications, assuming that all cell counts represented bacteria. As with the calculation of *T. denitrificans*-like 16S rRNA molecule numbers, total 16S rRNA molecule numbers were used and the Com product/OST product ratios were obtained from relative 16S rDNA quantification analyses. Total 16S rRNA molecule numbers were calculated as follows. Total RNA was quantified as described above. The RNA extraction efficiency of our phenol extraction protocol has been demonstrated to range from 52 to 66% and to be relatively constant for gram-negative bacteria (43). Therefore, we used a 60% extraction efficiency to calculate the real amount of total RNA based on the fluorescence measured. Neglecting tRNA and mRNA, which are minor components of the total RNA pool, the 23S and 16S rRNAs are present in a ratio of 2:1. Consequently, 16S rRNA quantities are just one-third of the total rRNA contents. Calculations for conversions between the mass and the number of copies of a 1,542-bp 16S rRNA were performed as follows (PCR Application Manual, 2nd ed., online version, Roche Molecular Biochemicals, Mannheim, Germany). To calculate molecular mass, the following formula was used: 1,542 bp  $\times$  340 Da/bp = 524,280 Da =  $5.24 \times 10^5$  Da. To calculate the number of picomoles per microgram, we used the formula  $10^6 \text{ pg}/\mu\text{g} \times [1 \text{ pmol}/(5.24 \times 10^5 \text{ pg})] = 1.91 \text{ pmol}/\mu\text{g}$ . To calculate the number of molecules per microgram, we used the formula  $[(6.022 \times 10^{23} \text{ molecules})/\text{pmol}] \times 1.91 \text{ pmol}/\mu\text{g} = 1.15 \times 10^{12} \text{ molecules}/\mu\text{g} = 1.15 \times 10^9 \text{ molecules}/\text{ng}$ .

**Evaluation of Q-PCR and Q-RT-PCR.** In order to evaluate the quality of our quantification protocols, the number of ribosomes per *T. denitrificans*-like bacterial cell was calculated and compared to literature data. Calculations were based on respective 16S rRNA molecule numbers divided by the specific numbers of *T. denitrificans*-like bacterial cells. A flow diagram, comprising the several steps, analyses, and calculations for the assessment of the copy numbers for 16S rRNA molecules and genes as well as the numbers of ribosomes per cell, is given in Fig. 2.

**Statistical analysis.** In order to test whether there was a significant difference ( $P < 0.05$ ) between results obtained by quantifications with and without preamplification, the Student *t* test was performed.

## RESULTS

**Environmental 16S rRNA content and total cell counts.** Environmental 16S rRNA content ranged from 0.04 to 1.41 ng ml of seawater<sup>-1</sup>, corresponding to  $4.65 \times 10^7$  to  $1.62 \times 10^9$  16S rRNA molecules ml<sup>-1</sup> (Table 1). The highest 16S rRNA content was observed in the photic zone (5 m). In enrichments, after 44 h (Bx21 and Bx23) or 88 h (Bx24) of incubation, 16S rRNA contents ranged from 3.23 to 7.74 ng ml<sup>-1</sup>, corresponding to  $3.71 \times 10^9$  to  $8.90 \times 10^9$  16S rRNA molecules ml<sup>-1</sup> (Table 1). Total cell counts in the water column ranged from  $0.27 \times 10^6$  to  $2.15 \times 10^6$  cells ml<sup>-1</sup>, with highest numbers occurring in the upper 30 m (Table 1). Total cell counts in enrichments ranged from  $1.66 \times 10^6$  to  $4.43 \times 10^6$  cells ml<sup>-1</sup>.

**Establishment of Q-PCR and Q-RT-PCR assays.** Using nucleic acids extracted from the Bx enrichments, quantification of *T. denitrificans*-like 16S rRNA molecules as well as 16S rRNA genes was successful without a preamplification step in a range from 8 to 50% relative abundance (Table 1). With 16S rRNA genes, the average PCR efficiency was slightly above 1 for the Com and the OST primer systems, respectively (Table 2). Similarly, with 16S rRNA, the average PCR efficiency was 0.8 for

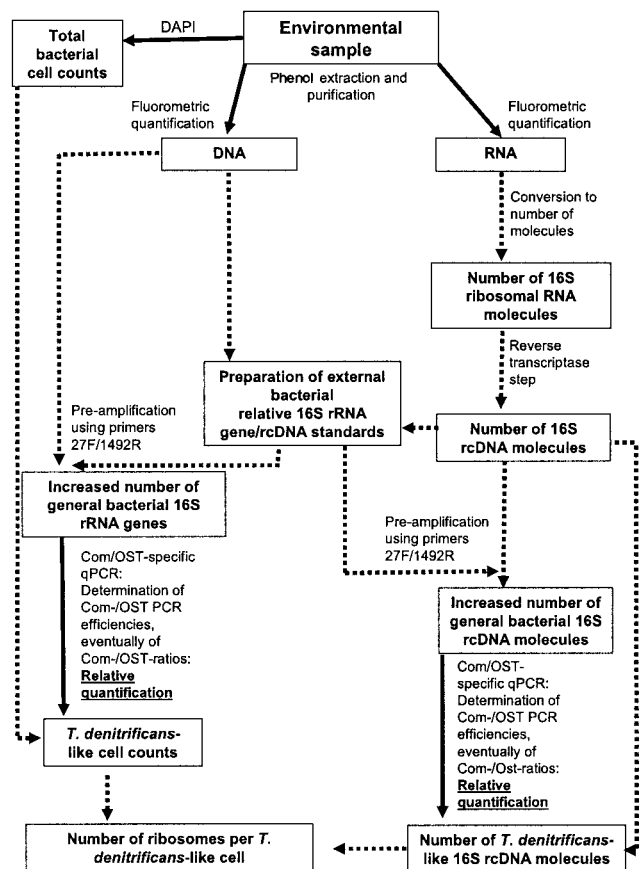


FIG. 2. Flow diagram of the culture-independent approach to assess the abundance of *T. denitrificans*-like bacteria. Solid lines depict those characteristics which have to be measured for our quantification approach. Dotted lines depict characteristics which have to be calculated in order to estimate the numbers of *T. denitrificans*-like cells, 16S rRNA molecules, and ribosomes. Detailed calculations are given in Materials and Methods.

the Com as well as the OST primer system. However, this protocol was not transferable to environmental samples, where the *T. denitrificans*-like organism was probably present in smaller amounts. Despite the fact that melting-curve temperatures indicated satisfactory amplification products for the bacterial

primer system, environmental 16S rRNA and 16S rRNA genes used as templates for real-time PCR sometimes resulted in impossible PCR efficiencies for the bacterial primer system or no amplification with the OST primer system (Fig. 3). Thus, our quantification protocol was further developed, resulting in a nested-PCR approach. Pre-amplification of 16S rRNA genes or 16S rcDNA with the 27F-1492R primer system before RT analyses with the Com and OST primer systems increased the molecule copy number  $3.5 \times 10^4$ -fold and resulted in improved PCR efficiencies for the Com as well as the OST primer system (Table 2). Moreover, Q-PCR and Q-RT-PCR was successfully applied to most of the environmental samples (Table 1). Regarding 16S rRNA analyses, the linearity of Com and OST amplification was given from quantities of  $7.8 \times 10^{10}$  to  $1.7 \times 10^3$  molecules (Fig. 4), with a correlation coefficient of 0.999. An upper quantification limit could not be evaluated. A comparison of the specific abundances obtained by real-time PCR without a pre-amplification step and those from analyses including a pre-amplification step is demonstrated in Fig. 5. Statistically, there was no evidence for a significant variation between results gained by both quantification procedures (Student's *t* test;  $P < 0.05$ ).

**Quantification of *T. denitrificans*-like bacteria after pre-amplification.** Quantification of *T. denitrificans*-like 16S rRNA molecules was successful in a range from  $7.1 \times 10^3$  to  $4.4 \times 10^9$  copies per ml or 0.002 to 49.7% relative abundance. Quantification of *T. denitrificans*-like 16S rRNA genes was successful in a range from  $9.0 \times 10^1$  to  $2.2 \times 10^6$  copies ml<sup>-1</sup> or 0.01 to 49.7% relative abundance (Table 1). The estimated number of ribosomes per environmental *T. denitrificans*-like cell ranged from 20 to 200 (mean value,  $100 \pm 70$ ) and increased during enrichments up to 2,000. This result demonstrated a 16- to 100-fold increase in comparison to levels in the seawater sample with which the enrichment was started (Table 1). OST products melted specifically at 77.3 to 77.4°C, and Com products melted at 82.7 to 82.8°C (Table 2). Other properties of the primer sets used for Q-PCR protocols with and without pre-amplification are also shown in Table 2.

## DISCUSSION

**Applicability of the approach.** Our approach, combining total cell counts, environmental RNA content, and real-time

TABLE 2. Properties of PCR and RT-PCR and the different primer sets used for Q-PCR protocols<sup>a</sup>

Target and primers	Reaction efficiency		Correlation coefficient of regression line		Melting temp (°C)		Detection limit of <i>T. denitrificans</i> -like nucleic acids ml <sup>-1b</sup>	
	Before	After	Before	After	Before	After	Before	After
16S rRNA gene								
Com	1.02 ± 0.02	0.99 ± 0.06	1.000 ± 0.000	0.999 ± 0.001	83.87 ± 0.16	82.73 ± 0.36		
OST	1.04 ± 0.15	0.97 ± 0.09	0.996 ± 0.003	1.000 ± 0.000	77.47 ± 0.14	77.44 ± 0.15	1.4 × 10 <sup>3</sup>	0.2
16S rRNA								
Com	0.81 ± 0.07	0.88 ± 0.08	0.999 ± 0.001	0.998 ± 0.002	82.52 ± 0.26	82.83 ± 0.33		
OST	0.84 ± 0.07	0.89 ± 0.08	0.998 ± 0.001	0.998 ± 0.002	77.38 ± 0.15	77.40 ± 0.17	7.4 × 10 <sup>4</sup>	20

<sup>a</sup> Before, results without a pre-amplification step, using nucleic acids extracted from Bx enrichments; after, results with a pre-amplification step. Mean values of three or more determinations ± standard deviations are given.

<sup>b</sup> Values are numbers of copies.

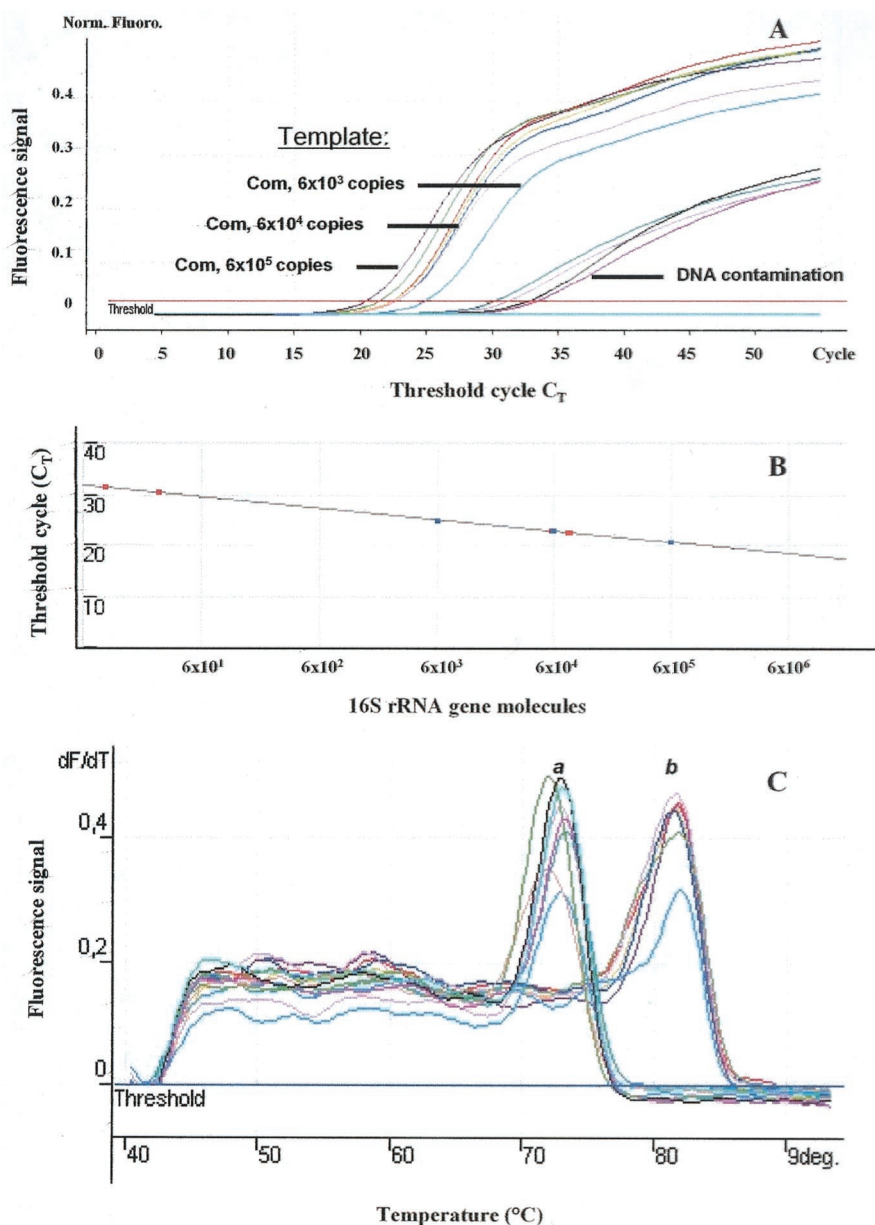


FIG. 3. Insufficient quantification of bacterial 16S rRNA genes from sample G6 (depth, 120 m) by real-time PCR (without preamplification). (A) Environmental DNA was used as the template in quantities of  $6.1 \times 10^5$  to  $6.1 \times 10^2$  bacterial chromosomes for the Com primer set, representing  $C_T$  values ranging from 20.6.0 to 25.1. Specific PCR products did not appear when environmental DNA was used as the template for the OST primer set. DNA contamination represented  $C_T$  values ranging from 30.4 to 33.6. Norm. Fluoro., normalized fluorescence. (B) Relation between the  $C_T$  and the apparent amount of 16S rRNA gene molecules by SybrGreen real-time PCR. Each point represents an amount of 16S rRNA gene molecules corresponding to the  $C_T$  value with the Com primer set. Values for standard molecules are represented as blue points, and those for samples are shown as red points. The correlation coefficient of the regression line was 0.999. PCR efficiency was 1.78. (C) Melting-curve analysis of online PCR signals. Peak *a* shows DNA contaminants melting at 72.0 to 73.0°C; peak *b* shows Com products melting at 81.5 to 82.0°C. deg., degrees.

PCR for the quantification of bacteria from the central Baltic Sea, was successfully applied for an uncultured epsilonproteobacterium related to *T. denitrificans*. It was applicable to enrichments as well as to oligotrophic seawater samples. The real-time PCR had at least a 5-log dynamic range of detection with the OST as well as the Com primer set. This detection range has already been observed by others (11). However, sometimes the bacterial 16S rRNA gene real-time PCR assay

was only log linear over 4 orders of magnitude. This finding accords with the data of Harms et al. (13), who found that results of the bacterial 16S rRNA gene real-time PCR assay were log linear over 4 orders of magnitude with DNA standards but that results with *Nitrospira* 16S rRNA genes, ammonia-oxidizing bacterium 16S rRNA genes, and *Nitrosomonas oligotropha*-like *amoA* genes were log linear over 6 orders of magnitude. This difference is probably due to the fact that

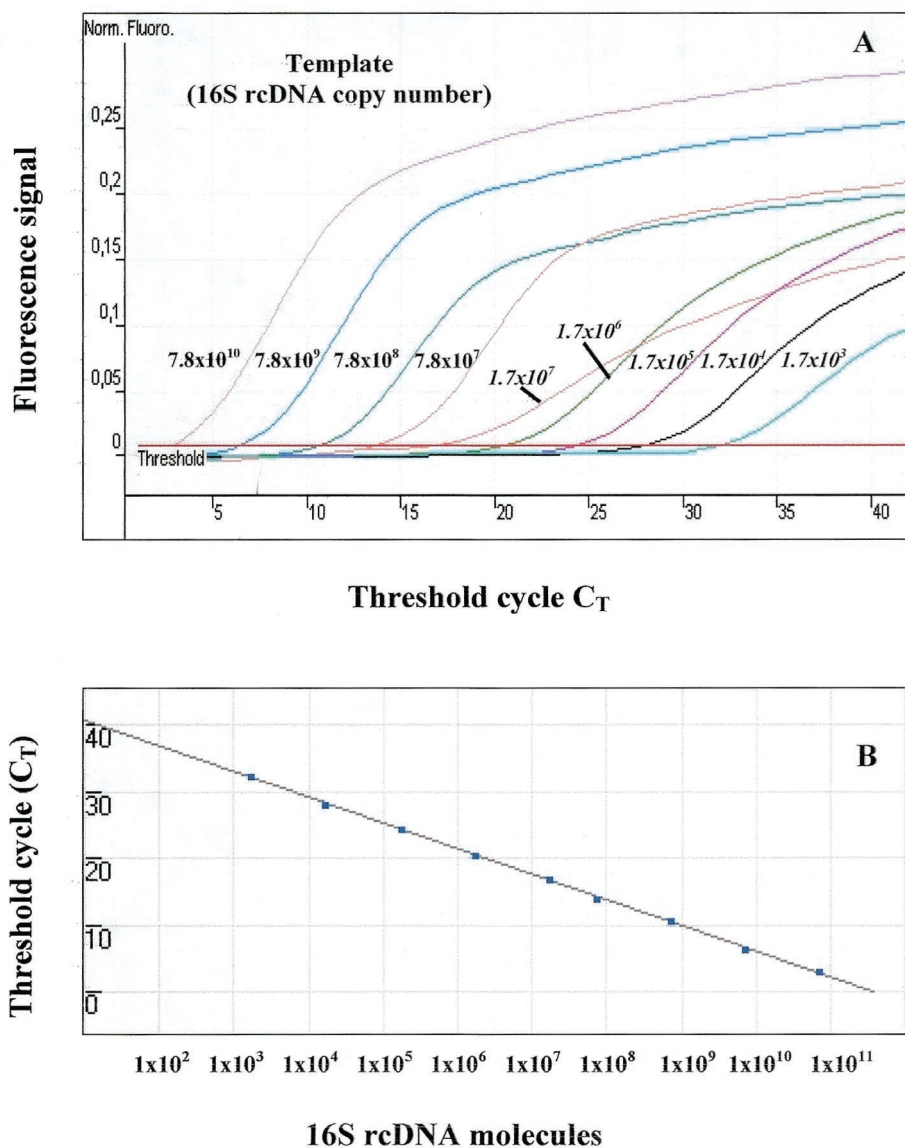


FIG. 4. Detection limits of environmental *T. denitrificans*-like 16S rRNA in sample G3 (50-m depth) from the Baltic Sea by real-time PCR. (A) Sensitivity of the Com and OST primer sets in quantifying *T. denitrificans*-like 16S rRNA. After RT-PCR with primer 1492R and preamplification with primers 27F and 1492R, 16S rcDNA was used as the template in quantities of  $7.8 \times 10^{10}$  to  $7.8 \times 10^7$  molecules for the Com primer set and  $1.7 \times 10^7$  to  $1.7 \times 10^3$  molecules for the OST primer set (values shown in italics), representing  $C_T$  values ranging from 3.0 to 32.2. Norm. Fluoro., normal fluorescence. (B) Relation between the  $C_T$  and the apparent amount of 16S rcDNA molecules obtained with SybrGreen real-time PCR. Each point represents an amount of 16S rcDNA molecules corresponding to the  $C_T$ -value with the Com and OST primer sets. The correlation coefficient of the regression line was 0.999. The PCR efficiency was 0.82.

primer systems of higher specificity are less sensitive to bacterial contamination. However, linear results over 7 orders of magnitude (from 7.5 ng down to 7.5 fg) have also been reported for a SybrGreen assay, a 5'-exonuclease assay, and a fluorescence resonance energy transfer assay used to detect *Brucella abortus* (27).

**Sensitivity and detection limits.** Contaminant genomic DNA is usually present in polymerase preparations and may lead to significant fluorescence in the no-template controls after about 35 cycles (1). In our experiments, this was indeed observed with the Com primer system, mostly with *E. coli* K-12 as a contaminant (data not shown), but usually not with the

OST primer set (data not shown). This may be due to the high specificity of the *T. denitrificans*-like primers employed. In an attempt to overcome the contamination problem, Corless et al. (6) applied several treatments, such as UV light, DNase I enzyme treatment, or restriction endonuclease digestion, but all treatments were accompanied with an associated decrease in sensitivity. In order to increase sensitivity, we applied the preamplification approach, which enriched the target for nested Q-PCR by more than 4 orders of magnitude. This approach resulted in an observable PCR product, but it must be noted that the additional PCR step may also increase the PCR biases. Furthermore, it should also be noted that our

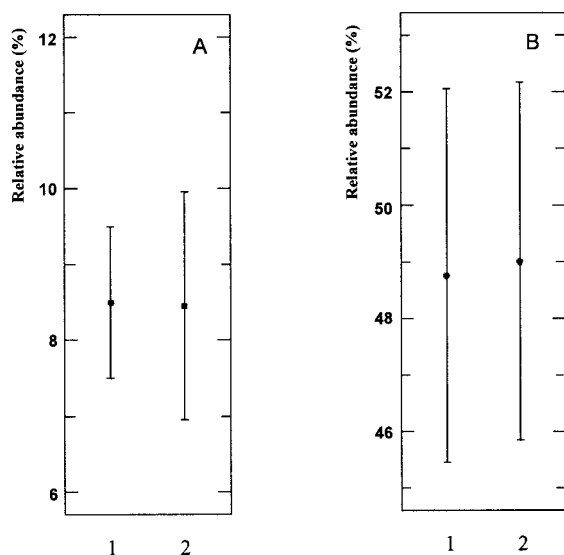


FIG. 5. Mean values of *T. denitrificans*-like 16S rRNA fractions (percent relative abundance) achieved from quantifications without (lane 1) and with (lane 2) a preamplification step. (A) Enrichment Bx21. The Student *t* test ( $P < 0.05$ ) showed no evidence for a significant variation between results gained by using both quantification protocols ( $P$  value, 0.96;  $n = 15$ ; degrees of freedom, 13). (B) Enrichment Bx24. The Student *t* test ( $P < 0.05$ ) showed no evidence for a significant variation between results gained by both quantifications protocols ( $P$  value, 0.83;  $n = 8$ ; degrees of freedom, 6).

enrichments consisting of at least 8% target DNA or RNA were successfully quantified without preamplification. However, quantification of these enrichments using a preamplification step did not yield significantly different results (Fig. 5), and community fingerprints with and without preamplification were also found to be highly similar (data not shown). Rodrigues et al. found that real-time PCR has a 6-log dynamic range of detection for DNA from pure cultures ( $10^2$  to  $10^7$  cells  $\text{ml}^{-1}$ ) but that it is less sensitive with soil samples (31). Moreover, their experiments suggested that the type of target gene sequence can also be an important factor in detection. They found different standard curves for the *fc*b-targeted gene and the 16S rRNA-targeted gene. Other factors affecting detection limits in PCR-based methods included the type and composition of the sample matrix, the type of target organism, the number and diversity of bacteria in the sample, the *Taq* polymerase used, and the DNA extraction protocol (22, 31). However, Teo et al. (38) suggested that a nested-PCR approach using a combination of cycle-limited conventional PCR and Q-PCR enables the detection of low-copy-number target DNA when it is present in a high background of nontarget DNA. In results similar to ours (Table 2), they found that the relative amplification efficiency of a nested-PCR approach equals that obtained with the target DNA and quantification standards. However, their experiments were based on human immunodeficiency virus type 1 DNA, and environmental samples can have high levels of chemical and genetic complexity not encountered in tissue or pure cultures (36). In general, detection limits for quantification by PCR range between 10 and 200 copies per reaction. For example, Baldwin et al. (2) detected aromatic oxygenase genes down to a detection limit of 200

copies with a SybrGreen assay, and Kolb et al. (19) detected 10 to 100 copies of the *pmoA* gene from methanotrophs in soil. However, quantification of DNA extracts from pure cultures of *S. aureus* in a LightCycler system using SybrGreen proved to be less sensitive (60 *nuc* gene copies/ $\mu\text{l}$ ) than using a fluorogenic TaqMan probe (6 *nuc* gene copies/ $\mu\text{l}$ ) (15). Considering the sample dilutions as well as preamplification, detection limits of our approach were much lower, being 20 copies for RNA and 0.2 copy for DNA  $\text{ml}$  of seawater $^{-1}$  (Table 2).

**PCR efficiencies.** Another source of error which may be encountered is varied PCR efficiencies. A perfect amplification, in which each target sequence is replicated during every cycle, would have an efficiency of 1.0. Bach et al. (1) detected correlations between fluorescence and starting copy number for three peptidase gene classes of the same high quality independently of a SybrGreen or a TaqMan assay. However, the PCR efficiencies of a SybrGreen assay, a 5'-exonuclease assay, and a fluorescence resonance energy transfer assay have been compared by Newby et al. (27). They found that the SybrGreen assay usually showed lower efficiencies, which was explained by the presence of the intercalating dye that can be inhibitory to PCR (46). Comparing PCR efficiencies for the *fc*b-targeted gene with those for the 16S rRNA-targeted gene, Rodrigues et al. (31) suggested that the target sequence can also be an important factor resulting in different efficiencies. In our studies, various PCR efficiencies were also detected (Table 2). To overcome this problem, each measurement was verified and standardized by its own domain-specific as well as *T. denitrificans*-like-organism-specific standardization derived from a dilution series of a corresponding environmental target 16S rDNA or 16S rRNA gene; i.e., each sample had its own set of standards and dilution series.

**Conversion of numbers of gene copies to specific cell numbers.** Some general aspects regarding quantification by real-time PCR must be taken into consideration. The final determination of bacterial load by real-time PCR in a multispecies community will be influenced by the variation in the number of rRNA operons in a given species (7). Further, the number of replication forks is directly related to the generation time, which in turn depends on the metabolic status of the bacteria at the time of sampling (17). Not knowing the exact number of copies of 16S rRNA operons in a given target species at the time of sampling represents a limitation to the assessment of bacterial numbers by real-time PCR based on the 16S rRNA gene (25). Thus, a DNA standard representing those bacteria most likely to predominate in any given habitat is important for a more accurate determination of the total bacterial load. For this reason, our external relative standardizations were specifically prepared for every sample quantified and solely based on its respective original community DNA or RNA, and they were as close to natural conditions as possible. Moreover, our approach combining different methods still allowed an estimation of numbers of taxon-specific cells and 16S rRNA molecules.

**Assessment of the number of ribosomes as an indicator of cell activity.** In general, the number of ribosomes per cell is considered to be a measure of the protein-synthesizing capacity of the cell and its growth potential or growth rate. Thus, the number of ribosomes per *T. denitrificans*-like cell was calculated to verify our quantifications. Calculation uncertainties because of various operon numbers or replication forks have

already been discussed above. An average 16S rRNA gene copy number per cell of 2.3 is assumed for “*Epsilonproteobacteria*” (18). The average 16S rRNA gene copy number of our investigated microbial community is unknown. However, in comparison to other data, our calculations may support our quantification assay: *E. coli*, growing at a maximal growth rate under laboratory conditions, can contain up to 72,000 ribosomes per cell (8). Dependent on its activity level, the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256 contained 200 to 2,000 ribosomes per cell (8). Investigating bacteria from four permanently cold marine stations, Sahm and Berninger (32) calculated numbers of 350 to 1,300 ribosomes per bacterial cell. Depending on the activity level, the number of ribosomes per *T. denitrificans*-like cell ( $\pm$  the standard deviation) ranged from 100 ( $\pm 70$ ) to 1,500 ( $\pm 500$ ). This appears to be reasonable for a marine taxon. These results indicate that our real-time PCR approach can be used for the assessment of both specific and relative abundances of uncultured marine bacteria.

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