LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 2, 2004, 365–373
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Optimization of DNA extraction for quantitative marine bacterioplankton community analysis

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Abstract

The coupling between the presence of predominant bacterial species and particular biogeochemical processes is of primary interest in current microbial ecology. Molecular methods such as microarrays and real-time polymerase chain reaction (PCR) may be used to estimate presence or expression of different genes (e.g., 16S rDNA and *nifH*) in environmental samples; however, to be quantitative, these methods require a reproducible and efficient DNA extraction protocol. Using picogreen DNA quantification, real-time PCR, and an internal DNA standard, we step-wise examined and optimized a protocol for DNA extraction from centrifuged or filtered seawater samples (vol. 2 to 300 mL). Sample volume had a pronounced effect on DNA extraction efficiency showing that comparison of samples with different volumes is problematic. The duration of enzyme treatment (lysozyme and proteinase K) significantly influenced the DNA extraction efficiency. Dissolved DNA contributed significantly to total DNA when extracted from small filtering volumes (<10 mL). Addition of a coprecipitant (yeast tRNA) improved the precipitation of low-concentration DNA from 13% to 89%. When tested on various seawater samples, as well as on isolates, the optimized extraction protocol was found to be highly reproducible with an average extraction efficiency for seawater samples of 92% and for isolates of 96%. The quantitative outcome and the high extraction efficiency of the presented extraction protocol will be of value to future studies based on DNA extracted from seawater samples.

Introduction

The coupling between population dynamics of single bacterial species and their phenotypic function is among the primary goals of current aquatic microbial ecology. So far, only few reports have succeeded in the establishment of such a connection between presence and function and often not in a strictly quantitative manner (e.g., Schramm et al. 1998; Casamayor et al. 2002). Theoretically, the detection of single species or groups should be feasible using the now widely applied fluorescence in situ hybridization (Amann et al. 1995).

Molecular biological methods that are based on nucleic acids extracted from marine microbial communities provide an alternative to single-cell labeling techniques and have proven extremely important in studies of species composition and distribution. Most of these are based on endpoint PCR and do not provide quantitative information. Alternative techniques such as real-time PCR or hybridization aiming at DNA or RNA have the potential to quantify population dynamics through the presence or expression of specific genes with a high sensitivity and resolution (Rehnstam et al. 1993; Guschin et al. 1997; Pinhassi et al. 1997; Roth 2002; Wawrik et al. 2002). Suzuki et al. (2001) demonstrated the rapid mapping of specific bacterioplankton groups in a large number of samples from Monterey Bay by using real-time PCR. A weakness of all those methods is that they require a highly efficient and reproducible extraction method, which delivers nucleic acids of high quality. Hence, the presence of certain genes/sequences in an environmental sample has, so far, not been quantifiable by these techniques but has been reported as relative numbers (e.g., Suzuki et al. 2001).

A wide array of different DNA extraction protocols are in use today for bacterial community analyses in aquatic microbial ecology. This is a major concern because differences in extraction efficiency might be reflected in the subsequent community analyses, as has recently been documented for soil communities (Stach et al. 2001). In an early paper by Fuhrman et al. (1988), an effort was made to adopt the then standard protocol for DNA extraction to environmental samples. Today modified versions of this protocol are commonly used when

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Acknowledgments

We thank Svend Binnerup, National Environmental Research Institute, Denmark, for providing the bacterial isolates, and Karin Holmfeldt and Henrik Hallberg for their assistance in filtering experiments. This work was supported by the Swedish science councils VR (621-2001-2814) and FORMAS (2002-1300) and by the EC through contract EVK3-CT-2002-00078 "BASICS" to ÅH.

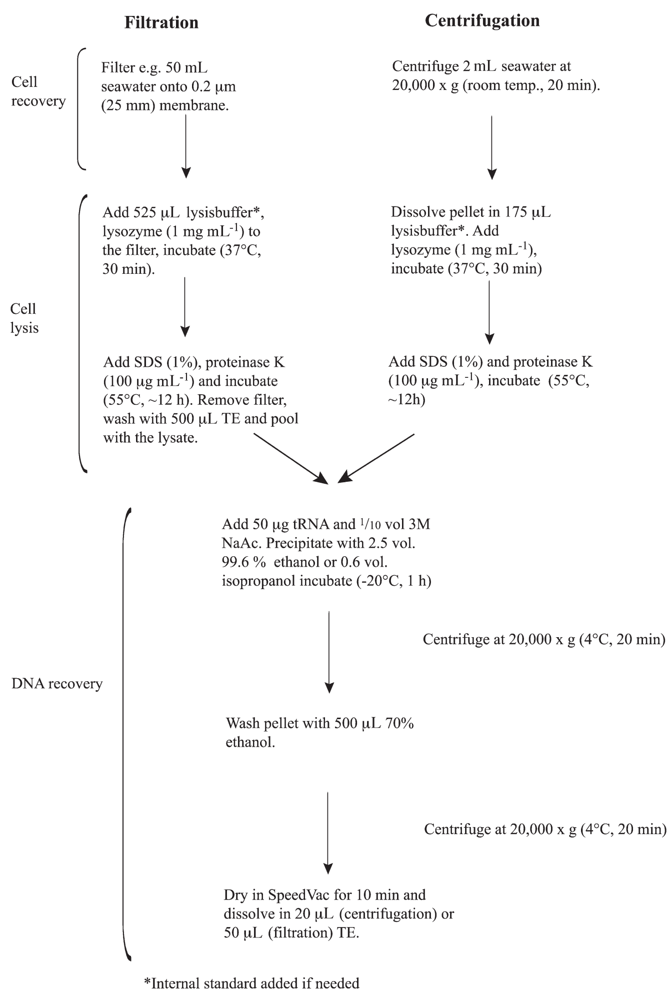

Fig. 1. The DNA extraction protocol optimized for obtaining DNA from seawater samples. Final concentrations are shown in parentheses.

extracting nucleic acids from marine waters (e.g., Moeseneder et al. 1999; Dumestre et al. 2001; Weinbauer et al. 2002). The DNA extraction efficiency was reported by Fuhrman et al. (1988) to be 25% to 50% and by Weinbauer et al. (2002) to be 20% to 60%, but it is not reported in most studies.

In the present study, we sought to optimize the different steps in an extraction protocol based on the Fuhrman et al. (1988) study. Three particular phases in the protocol were optimized: cell recovery, cell lysis, and DNA recovery. In addition, the effect of phenol/chloroform extraction as well as the influence of sample size on the DNA extraction efficiency was examined. Fluorometric quantification of total DNA was combined with real-time PCR quantification of specific gene counts and an internal standard to optimize the extraction protocol. This was then applied to seawater community samples and to a selection of bacterial isolates. The optimized extraction protocol was found to be highly reproducible and had an average extraction efficiency for seawater samples of 92% and for isolates of 96%.

Materials and procedures

The optimized DNA extraction protocol is presented in Fig. 1. Materials used for the assessment and detailed information on buffers and solutions for the optimized protocol are given below. The different steps in the protocol were optimized consecutively using seawater collected from the southern Baltic Sea (near Kalmar, Sweden) at various times between March 2002 and March 2003. For each optimization step, water from one sampling was used. DNA recovery optimization was made using genomic isolate DNA. For lysis and extraction efficiency experiments, isolates (*Escherichia coli* K12, ATCC 10798; *Bacillus cereus*, ATCC 10987; *Bacillus subtilis*, ATCC 23857; *Agrobacterium tumefaciens*, ATCC 33970; *Xanthomonas campestris,* ATCC 33913) as well as seawater samples from the Baltic Sea and from the Mediterranean (near Banyuls, France) were used. Where nothing else is stated, DNA evaluation was made with picogreen (Molecular Probes) and real-time PCR (described later). For centrifugations, an Eppendorf 5417 C (rotor F 45-30-11) was used at maximum speed (20000 *g*), and where nothing else is stated 2-mL polypropylene tubes (Sarstedt) were used.

Cell recovery. To evaluate cell recovery, seawater samples were either centrifuged (20000 *g*; 0, 5, 10, 20, 30, or 60 min, room temp.) or filtered onto 25 mm dia, 0.2 µm Supor®-200 polyethersulfone membrane filters (PALL Corporation). Centrifuged bacteria were stained with SYTO 13 (Molecular Probes) and counted on a FACSCalibur flow cytometer (Becton Dickinson) according to Gasol et al. (2000). Fluorescent beads (True counts, Becton Dickinson) were used as standard in each sample. The cell recovery as a result of different filtration volumes was determined from the amount of extracted DNA using picogreen and real-time PCR.

Cell lysis. A volume of 175 µL or 525 µL lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 9.0) was added to the centrifuged (C) and filtered (F) samples, respectively. A volume of 3.5 µL (C) or 11 µL (F) lysozyme (1 mg mL^{-1}, final concentration (conc.), Sigma-Aldrich) was added and incubated at 37°C. Then 20 µL (C) or 60 µL (F) sodium dodecyl sulfate (SDS) (1%, final conc., Sigma-Aldrich) and 1 µL (C) or 3 µL (F) proteinase K (100 µg mL^{-1}, final conc., Roche Applied Science) were added, and the samples were incubated at 55°C (Moeseneder et al. 1999). The filter samples were rotated in a hybridization oven during incubation.

To evaluate the time of enzyme treatment, 2 mL seawater samples were used and extracted according to Fig. 1 but without the addition of tRNA and with variable incubation times for lysozyme (0, 15, 30, and 60 min) and proteinase K (30, 60, 120 min, and overnight).

For the evaluation of cell disruption using beads or enzymatic treatment, centrifuged (2 mL) or filtered (50 mL) seawater samples were used. For cell disruption using beads, 1 mL TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 100 µL SDS (0.9%, final con.), and 1.3 g 0.1-mm glass beads (BioSpec Products, Inc.)

were added, and the samples were vortexed for 5 min at full speed (2750 rpm, Geine2, Scientific Industries). To remove the beads with as little sample loss as possible, the filter was removed, the sample transferred with the beads to a 1-mL glass-fiber-filter–stoppered syringe, placed in a 15-mL Falcon tube and centrifuged (1 min, 5000 *g*, room temperature, Beckman Avanti J-25, rotor JA 14). The beads were washed with 0.5 mL TE and DNA was precipitated according to Fig. 1 without the addition of tRNA. Evaluation was made with and without beads and with and without lysozyme and proteinase K treatments (according to Fig. 1 without the addition of tRNA). In addition, DNA from filtered seawater samples (50 mL) was extracted with the DNeasy Tissue kit (Qiagen) according to the protocol for Gram-negative bacteria.

Lysis efficiency of the optimized protocol was calculated from counts of intact cells before and after the lysis step using SYBR Green I (Molecular Probes) according to Noble and Fuhrman (1998).

DNA recovery. Fifty milliliters Baker's yeast tRNA (50 µg per sample, Roche Applied Science, nr 109 509) was used as coprecipitant (Ausubel et al. 2002; Wang et al. 2002). DNA was precipitated with 1/10 volume of 3 M NaAc, pH 5.2, and 2.5 volume 99.6% ethanol or 0.6 volume isopropanol (Sigma-Aldrich). The precipitation efficiency for the alcohols was found to be equal (data not shown). The pellet was washed with 70% ethanol, dried in a SpeedVac® Concentrator (Savant, Westshore Technologies), dissolved in TE, and kept at 4°C.

To evaluate the loss of DNA during phenol/chloroform extraction, 200 µL genomic isolate DNA (in total 22.5 or 225 ng) was extracted and subsequently quantified. The loss was quantified by comparing the DNA amount before and after the extraction using real-time PCR. To test if DNA of a sufficient quality could be obtained without phenol/chloroform extraction, various volumes (50, 100, and 400 mL) of a seawater-based laboratory diatom/dinoflagellate bloom were filtered and lysed according to Fig. 1. Then one volume of chloroform/isoamylalcohol, 24:1 (Sigma-Aldrich) was added, and the sample was vortexed and centrifuged (5 min). The aqueous phase was transferred to a new tube, extracted with one volume of phenol/chloroform/isoamylalcohol, 25:24:1, (Sigma-Aldrich), and then vortexed and centrifuged (5 min) according to Ausubel et al. (1994). Then DNA was ethanol precipitated according to Fig. 1 without the addition of tRNA. The effect of phenol/chloroform extraction on the quality/purity of the resulting DNA was evaluated by comparing real-time PCR amplification of samples extracted with and without phenol/chloroform treatment.

Total DNA and 16S rDNA quantification. Total DNA was quantified using the picogreen® dsDNA quantitation kit (Molecular Probes) and a FLUO star (BMG LabTechnologies) fluorometer for microplates. 16S rDNA was quantified by real-time PCR using the TaqMan (Applied Biosystems) bacteria-specific BACT2 primer/probe set developed by Suzuki et al. (2000): BACT1369F (1000 nM, final con.), PROK1492R (500 nM, final con.), and the FAM-labeled probe TM1389F (100 nM, final con.). A linearized pGEM®-T Easy plasmid (Promega) with a 16S rDNA insert (total 4452 bp) was used as standard DNA for BACT2 real-time PCR. In the final DNA extraction efficiency experiments, a linearized plasmid (pBSK, pBluescript II SK, 1.7 ng µL^{-1}, Stratagene) was used as an internal standard (8 ng for centrifuged samples and 34 ng for filtered samples). This standard was quantified by real-time PCR using the following primer/probe set: forward primer pBSKf (GTT GTA AAA CGA CGG CCA GT, 300 nM, final concentration), reverse primer pBSKr (GGC CCG GTA CCC AAT T, 900 nM, final concentration), and probe pBSKp (VIC-AGC GCG CGT AAT ACG ACT CAC TAT AGG-TAMRA, 250 nM, final concentration). Triplicate reactions were run for each sample in an ABI PRISM® 7700 Sequence detection system (Applied Biosystems). PCR conditions for both primer/probe sets were according to Suzuki et al. (2000) with the use of Platinum Taq DNA polymerase (Invitrogen).

Assessment

The extraction optimization was divided into 3 phases (Table 1). Each of these was optimized for highest DNA recovery. Results in the assessment are presented as ng DNA or pg 16S rDNA per mL seawater.

Cell recovery—Filtration volume. Different volumes of seawater were filtered through membrane filters, which were stored at –20°C until DNA extraction. Extraction was done according to Fig. 1 without the addition of tRNA. An increased sample volume did not yield a corresponding linear increase in recovered DNA (Fig. 2a). At small volumes (1 to 5 mL seawater), 19 ng DNA mL^{-1} and 40 pg 16S rDNA mL^{-1}, were recovered, whereas at high volumes (100 to 300 mL seawater), only 3 ng DNA mL^{-1} and 17 pg 16S rDNA mL^{-1} were recovered. These observations could possibly arise if dissolved DNA from small volumes of seawater adsorbs (Turk et al. 1992) and saturates chemical DNA-binding sites on the filter. To evaluate this, seawater was filtered sequentially through a polycarbonate filter (0.2 µm, 47 mm) to remove bacteria and particles and then through a polyethersulfone membrane filter (0.2 µm, 25 mm). DNA extracted from the membrane filter was defined as dissolved DNA. Due to the unknown adsorption of DNA to the polycarbonate pre-filter, we presumably underestimated the true concentration of dissolved DNA in the sample. Filtration of less than 10 mL yielded an unexpected large amount of dissolved DNA per mL seawater; up to 10 ng DNA mL^{-1} and 74 pg 16S rDNA mL^{-1} (Fig. 2b).

Filtration allows large amounts of DNA to be harvested. However, filtering different sample volumes does not yield the same DNA amount per mL seawater, making comparisons between such samples difficult. Another issue to consider is that the contribution of dissolved DNA is significant when filtering small volumes.

Centrifugation time. Two milliliters seawater samples were centrifuged for different periods of time. Bacteria were enumerated in the top milliliter of the supernatant and in the pel-

Table 1. A summary of questions asked and results obtained during the evaluation process

Questions asked	Optimization test	Samples*	Results
Cell recovery			
Can most cells be recovered from environmental samples by filtration?	DNA conc. measured by picogreen and real-time PCR	Seawater: 0.5, 1, 5, 10, 50, 100, 150 and 300 mL (F)	Incomparable results with different sample volumes
Can most cells be recovered from environmental samples by centrifugation?	Cell recovery measured by flow cytometry	Seawater: 2 mL (C)	Optimal recovery (99%): 20 min, 20,000*g*, room temp.
Cell lysis			
Does enzymatic treatment lyse most of the cells?	Lysis efficiency was determined by microscopy	2 mL (C), 100 mL (F) and isolates	Yes, 96%-100%
What is the effect of different incubation times of lysozyme and proteinase K on cell lysis?	DNA conc. measured by picogreen and real-time PCR	Seawater: 2 mL (C)	Optimal time for lysozyme: 30 min and for proteinase K: ~12 h
Does cell disruption with beads yield a high recovery?	DNA conc. measured by picogreen and real-time PCR	Seawater: 2 mL (C) and 50 mL (F)	Yes, in extraction from filter
Does a commercial spin column yield a high recovery?	DNA conc. measured by picogreen and real-time PCR	Seawater: 50 mL (F)	No
DNA recovery			
What is the efficiency of ethanol precipitation?	DNA conc. measured by real-time PCR	Genomic isolate DNA	Low, 85% to 90% loss of DNA
Does a coprecipitant help to avoid precipitation losses?	DNA conc. measured by real-time PCR	Genomic isolate DNA	Yes, improve recovery of DNA >6-fold
Are there a significant DNA loss in the phenol/chloroform extraction?	DNA conc. measured by real-time PCR and end-point PCR	Genomic isolate DNA	Yes, 30% to 40% loss
Can direct ethanol precipitation without phenol/chloroform extraction yield DNA suitable for real-time PCR?	DNA conc. measured by real-time PCR	Nutrient amended seawater culture 50, 100, and 400 mL (F)	Yes (with these samples)
DNA extraction efficiency of the final protocol	Extraction efficiency measured using an internal DNA standard and real-time PCR	Seawater: 2 mL (C), 100 mL (F). and isolates	Average 92% (isolates) and 96% (seawater)

*F = filter; C = centrifugation.

let, which was resuspended in the remaining volume. Optimal centrifugation time was found to be 20 min, where 99% of the cells were recovered in the pellet (Table 2). Notably, the number of cells in the pellet decreased when centrifuging greater than 30 min, possibly due to cell lysis. Centrifugation allows for efficient harvesting of cells from small sample volumes. Further, problems with contaminating dissolved DNA, which is inherent in filtration based extraction, are circumvented.

Cell lysis—Time of enzyme treatment. Two milliliter seawater samples were centrifuged (20 min, 20000 *g*, room temperature), and the pellets were dissolved in 175 µL lysis buffer. An array of samples was arranged so that increasing time of lysozyme treatment could be tested in parallel with increasing time of proteinase K treatment. Precipitation was done according to Fig. 1 without the addition of tRNA. As shown in Fig. 3, optimal cell lysis was obtained by 30 min lysozyme treatment and ~12-h proteinase K treatment. It is noteworthy that a prolonged lysozyme treatment decreased the DNA yield.

Lysis efficiency. Isolate cultures (10^6 to 10^7 bacteria) and seawater (2 mL centrifuged and 100 mL filtered) samples were tested for lysis efficiency (Table 3). The samples were lysed with lysozyme, SDS, and proteinase K according to Fig. 1. After incubation, intact cells were counted in the microscope. In the filter samples, both cells in the supernatant and on the filter (stained directly on the membrane filter) were counted. For all seawater samples and isolates, more than 95% of the cells were lysed after overnight incubation. Even the Gram-positive bacteria (*B. cereus* and *B. subtilis*) had a lysis efficiency of more than 98%.

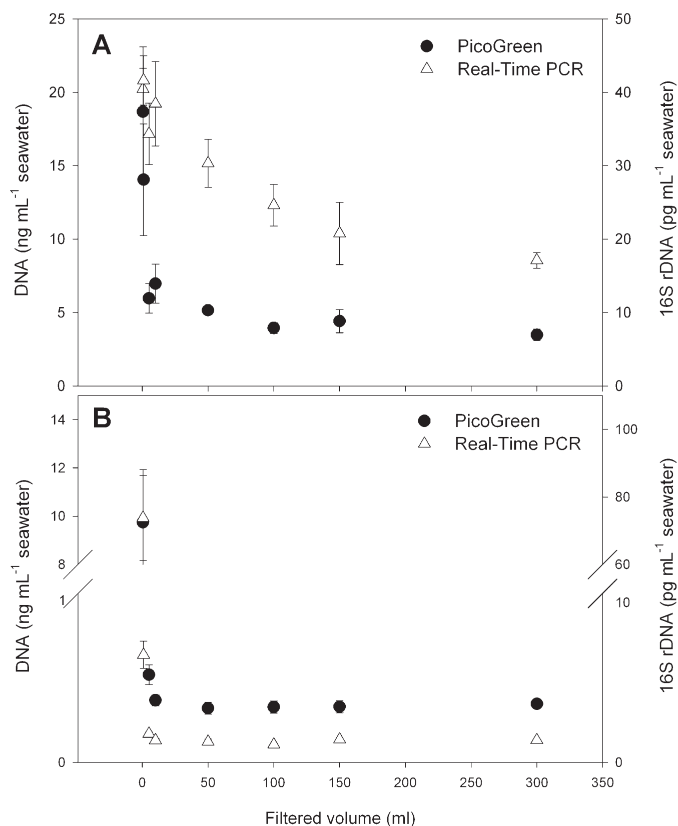

Fig. 2. Cell recovery. Total DNA (A) and dissolved DNA (B) recovered from different filtered volumes of seawater quantified with picogreen (n = 3, ± SE) and real-time PCR (n = 3, ± SE). For dissolved DNA extraction, seawater was filtered through a polycarbonate filter (0.2 µm) and onto a polyethersulfone membrane filter (0.2 µm) from where DNA was extracted.

Cell disruption using beads, enzyme treatment, or spin column. Cell disruption using beads and enzyme treatment were tested on centrifuged (2 mL) and filtered (50 mL) seawater samples. Samples were treated with or without beads and with or without an initial lysozyme and proteinase K step according to Fig. 1 (without the addition of tRNA). DNA from filtered samples was also extracted with the DNeasy Tissue kit (Qiagen) according to the protocol for Gram-negative bacteria. As shown in Fig. 4, enzyme treatment yielded the highest recovery for the centrifuged samples. However, for filter samples the use of beads produced a slightly higher recovery. A possible explanation is that at low DNA concentrations (centrifuged samples) the adsorption to beads is an important DNA loss while for larger samples (filters) the loss is insignificant. Spin columns were less efficient than either other method. This might be due to a lower lysis efficiency and a DNA adsorption to the column. Adding genomic DNA (22.5 or 225 ng) directly to the spin column resulted in a 30% DNA loss (data not shown).

DNA recovery—Ethanol precipitation. Precipitation of 100 ng genomic isolate DNA (2 ng µL^{-1}), which roughly corresponds to the DNA amount extracted from 20 to 30 mL seawater (Fig. 2a), yielded a DNA recovery of only 13% measured with real-time PCR (Fig. 5). To improve the precipitation efficiency, tRNA from yeast cells was added as coprecipitant (Ausubel et al. 2002; Wang et al. 2002). Addition of 50 µg tRNA (per sample) yielded an almost 90% DNA recovery while addition of 100 µg tRNA or more affected the real-time PCR assay. The apparent decrease was probably due to inhibition of the real-time PCR as was documented by unbiased amplification of diluted samples (data not shown). Amplification controls were performed on tRNA as well as on DNase-treated tRNA using real-time PCR and endpoint PCR. No amplification was ever observed (data not shown); however, we recommend always using DNase-treated tRNA.

Phenol/chloroform extraction. In the phenol/chloroform evaluation, 200 µL genomic isolate DNA (22.5 or 225 ng) was extracted and ethanol precipitated according to the optimized protocol. The phenol/chloroform extraction caused a 37 ± 2% loss of DNA (n = 3, data not shown). The necessity of phenol/chloroform extraction in the protocol was tested on "worst case scenario" samples. A dense (25 µg chl *a* L^{-1}) laboratory diatom/dinoflagellate bloom was generated in nutrient amended seawater and DNA was extracted from filtered samples (50, 100, and 400 mL; Fig. 6). Even when extracted from a large sample volume, the obtained DNA was sufficiently clean for real-time PCR, and the efficiency of the PCR was the same as for the phenol/chloroform extracted samples (data not shown). The difference in relative PCR yield between samples with and without phenol/chloroform extraction corresponded roughly to the 37% loss of DNA caused by the phenol/chloroform extraction itself (Fig. 6). This indicates, that at least in this case, DNA sufficiently pure for real-time PCR could be obtained without a phenol/chloroform extraction.

Extraction efficiency—After optimization of each step in the protocol, the total DNA extraction efficiency was determined. Initially, we sought to estimate the efficiency by relating total DNA in sonicated samples to extracted DNA by means of picogreen as described in Weinbauer et al. (2001). However, incomplete cell lysis necessitated the use of lysozyme in addition to sonication, which in turn, significantly biased the picogreen measurements (data not shown).

Instead, the overall extraction efficiency was determined in two steps. First, it was verified that the protocol provided almost complete cell lysis (96% to 100%, Table 3) for all sam-

Table 2. Cell recovery. Number of cells in pellet and supernatant after different centrifugation times (n = 3, ± SE).

	0 min	5 min	10 min	20 min	30 min	60 min
Supernatant (× 10^4 cells mL^{-1})	94.7 ± 2.9	8.1 ± 3.5	1.7 ± 0.6	0.7 ± 0.04	0.4 ± 0.01	0.2 ± 0.03
Pellet (× 10^5 cells mL^{-1})	0	10.8 ± 0.4	8.3 ± 0.2	10.4 ± 0.03	10.0 ± 0.3	7.0 ± 0.2

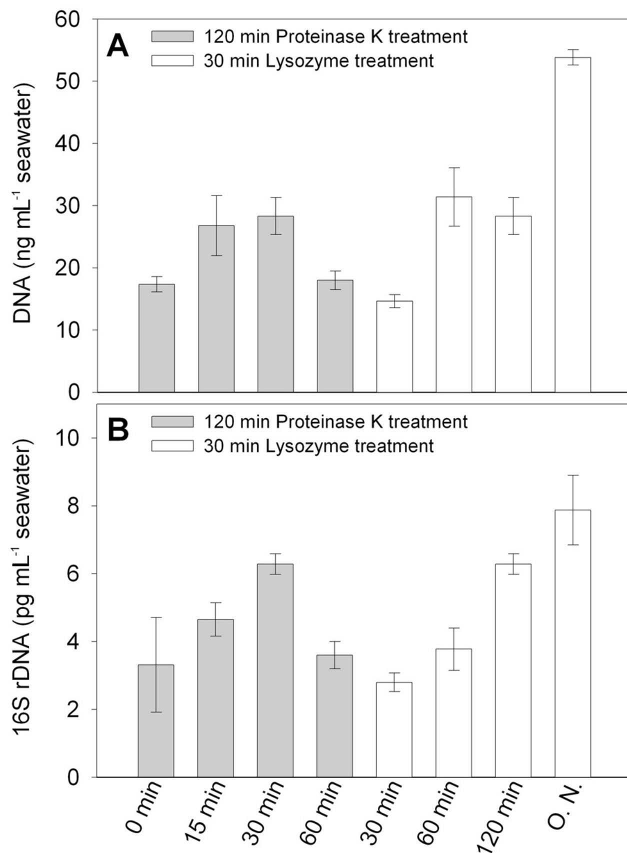

Fig. 3. Cell lysis. Recovered DNA using different treatment times for lysozyme and proteinase K. (A) Total DNA concentration measured with picogreen (n = 3, ± SE); (B) 16S rDNA measured with real-time PCR (n = 3, ± SE).

ples. Then the extraction efficiency and recovery was determined by adding an internal DNA standard (pBluescript plasmid) to the samples along with lysis buffer. Triplicate samples from five different isolates (both Gram-positive and Gram-negative) and from seawater samples from two different locations (both centrifuged and filtered samples) were extracted. For each of the triplicate samples, a reference sample (containing everything except the internal standard) was extracted in the same way, and after finishing the extraction was supplemented with an amount of internal standard like the sample. This was done to account for factors in the DNA extract affecting the real-time PCR quantification of the standard. Samples and reference samples were run in the same PCR batch (triplicates of each sample) and the extraction efficiency was calculated (pg internal DNA standard$_{sample}$/pg internal DNA standard$_{reference}$). The optimized extraction protocol was found to have an average extraction efficiency for seawater samples of 92% and for isolates 96% (Table 3).

Table 3. Isolates and community samples, centrifuged (C) and filtered (F)

Sample	Lysis efficiency (%)*	Extraction efficiency (%)†
Escherichia coli (C)	100	98 ± 12
Xanthomonas campestris (C)	100	108 ± 4
Agrobacterium tumefaciens (C)	100	97 ± 11
Bacillus subtilis (C)	100	108 ± 8
Bacillus cereus (C)	98	71 ± 16
Average (*isolates*)	100	96 ± 10
The Baltic (C)	96	100 ± 6
The Baltic (F)	100	92 ± 13
Banyuls (C)	99	102 ± 11
Banyuls (F)	100	75 ± 6
Average (seawater)	99	92 ± 9

*Samples lysed with lysozyme, SDS, and proteinase K. Intact cells were counted in the microscope (n = 3).
†Samples extracted using the optimized protocol and an internal DNA standard. Efficiency measured with real-time PCR (n = 3, ± SE). The reported SE was calculated from the reference sample and the efficiency sample: $SE = \sqrt{SE_1^2 + SE_2^2}$.

Discussion

In the present work, we have optimized a commonly used protocol for DNA extraction from seawater samples based on the original study by Fuhrman et al. (1988). The different steps in the protocol were successively optimized for maximum yield of DNA suitable for real-time PCR amplification. The optimized protocol was highly repeatable with an average standard error of 10% and an average extraction efficiency of 92% to 96%. This is, to our knowledge, among the highest efficiencies reported from seawater samples.

The extraction efficiency was measured using an internal DNA standard, which was extracted along with the sample and quantified by real-time PCR. Different kinds of internal standards for extraction and real-time PCR have been used in other studies (e.g., Widada et al. 2001; Brunk et al. 2002). Alternatively, DNA extraction efficiency could have been estimated using the number of cells in the sample (as done by Fuhrman et al. 1988); however, due to the variability of methods for enumerating the number of DNA-containing cells (Zweifel and Hagström 1995) and in the DNA content per cell for marine bacteria (e.g., 2.6 to 5.75 fg cell^{-1}; Fuhrman and Azam 1982, Jeffrey et al. 1996), we chose to use an internal DNA standard to calculate the efficiency of the optimized protocol.

The extracted DNA was quantified by real-time PCR and picogreen. The ratio between these two measures would presumably be rather variable between samples because of differences in the number of 16S rDNA copies bacterium^{-1} between species (Fogel et al. 1999) and differential loss of DNA during extraction. Knowledge of the exact size of the standard used in real-time PCR makes it possible to convert the outcome from pg 16S rDNA mL^{-1} seawater to 16S rDNA copies mL^{-1} seawater and relate this to the total amount of extracted DNA. Pooling all our data, we found an average ratio of 1.7 ± 0.3 16S rDNA copies per fg DNA (n = 30; data not shown), which is relatively

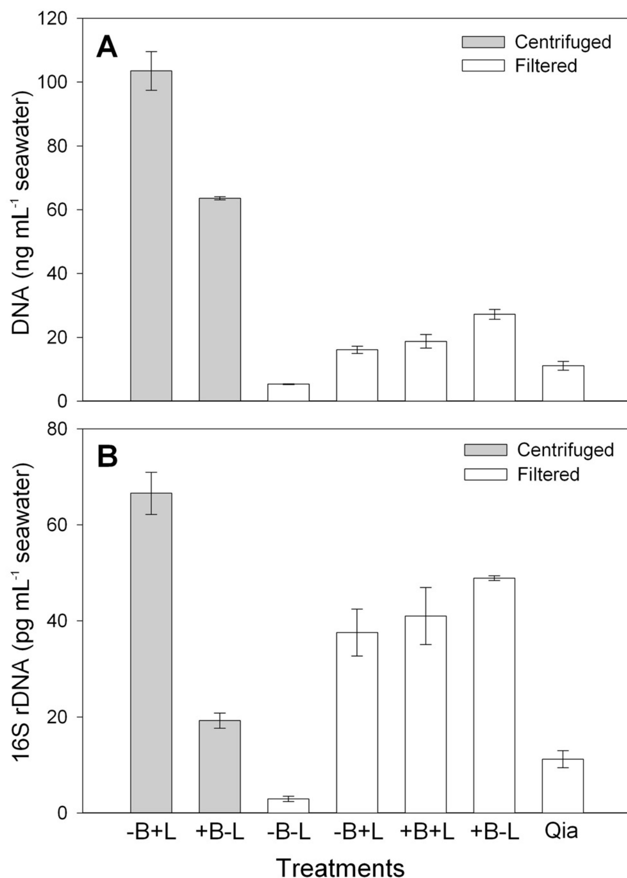

Fig. 4. Cell lysis. Cell disruption using beads versus enzymatic treatment. Centrifuged (2 mL) and filtered (50 mL) samples. Extraction was made with (+B) and without beads (–B), with (+L) and without lysozyme and proteinase K (–L). Filtered samples extracted with Qiagen spin column (Qia). (A) Total DNA concentration measured with picogreen (n = 3, ± SE). (B) 16S rDNA measured with real-time PCR (n = 3, ± SE).

close to the ratio of 0.7 to 1.5 copies per fg DNA, which may be calculated from literature values of DNA content cell^{-1} (2.6 to 5.75 fg cell^{-1}; Fuhrman and Azam 1982; Jeffrey et al. 1996) and the average number of 16S rDNA copies of bacteria (3.8; Fogel et al. 1999). This indicates that for our samples the number of 16S rDNA copies could have been roughly estimated from the total amount of extracted DNA using literature values for DNA cell^{-1} and 16S rDNA copy number cell^{-1}.

The study highlighted that when comparing quantitative samples, it is recommended to filter the same sample volume because the volume may influence the DNA extraction efficiency. Further, when filtering small sample volumes, a significant fraction of the extracted DNA might originate from dissolved DNA, which can influence the outcome of subsequent community analyses. This bias may be avoided by harvesting cells using centrifugation. Thus far, the relatively low concentration of bacteria in seawater samples combined with inefficient DNA extraction protocols has made filtration of large sample volumes a necessity. However, owing to its high efficiency,

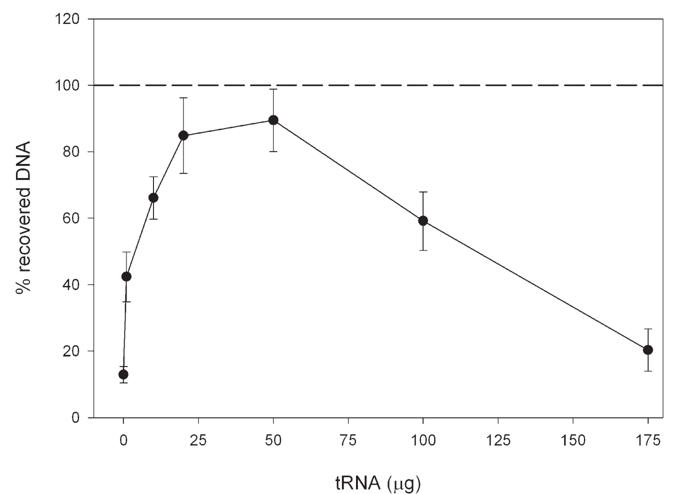

Fig. 5. DNA recovery. Recovered DNA (%) with increasing amount of tRNA used as co-precipitant. 16S rDNA was measured with real-time PCR (n = 3, ± SE).

the present protocol allows for 2 mL samples to be centrifuged and extracted. Recently, Long et al. (2001) found that although bacterial community composition varied at the microscale, no difference in community composition was observed between large (liters) or small (milliliters) volume samples. Similar observations were reported by Kirchman et al. (2001).

One advantage of centrifugation is the reduced handling of samples relative to filtration; however, a major concern is that the extraction of small DNA amounts leads to a significant DNA loss during the ethanol precipitation step. By precipitating genomic DNA, at a concentration comparable to the one in extracted 20 to 30 mL seawater samples, it was shown that the addition of a coprecipitant (tRNA) reduced the DNA loss during precipitation more than 6-fold.

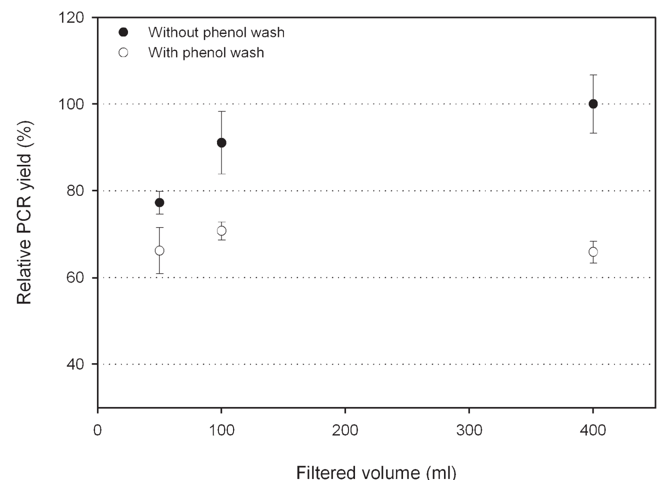

Fig. 6. DNA recovery. Recovered DNA from different sample volumes of a laboratory phytoplankton bloom extracted with and without phenol/chloroform. DNA obtained per mL seawater presented as percentage of peak value measured with real-time PCR (n = 3, ± SE)

During the optimization of the protocol, it was found that DNA sufficiently pure for real-time PCR and for endpoint PCR (data not shown) could be obtained without phenol/chloroform extraction. This was observed for isolates, various seawater samples, and even for a laboratory phytoplankton bloom. Therefore, the phenol/chloroform extraction step was omitted from the protocol. It cannot be denied that the protein-removing phenol/chloroform step may be necessary for some samples, and it is well-known that many researchers use phenol/chloroform extraction or a spin column (or both) for the purification of DNA (e.g., Suzuki et al. 2001). In such a case, an option is to add an internal standard and then do phenol/chloroform extraction and/or use a spin column. Whereas a DNA loss of approximately ≈30% to 40% for each of these steps should be expected, this loss may be quantified using the internal standard.

In the present optimization, the overall efficiency of the extraction process was quantified using an internal DNA standard and real-time PCR. Efficiencies of DNA extraction protocols have been estimated before (Fuhrman et al. 1988; Weinbauer et al. 2002); however, by combining high efficiency and repeatability with the option to use an internal standard for monitoring potential DNA loss during extraction, the present protocol provides a quantitative outcome. The ability to quantitatively extract DNA is a first step toward a quantification of single genes in seawater.

Comments and recommendations

Researchers in ecology using molecular techniques face important choices when adapting a nucleic acid extraction protocol for environmental samples. Few protocols are developed for work with bacteria, other than laboratory strains in defined media; hence, often leading to a highly inefficient DNA recovery. In the present study, we optimized a DNA extraction protocol using small sample volumes (2 to 300 mL) as opposed to more cumbersome samples sizes (liters) often used in previous protocols (Fuhrman et al. 1988; Moeseneder et al. 1999; Weinbauer et al. 2002). Our main focus was to optimize the yield of DNA while maintaining a DNA quality suitable for real-time PCR. Compared to the original protocol of Fuhrman et al. (1988), the DNA extraction efficiency was improved from 25% to 50% to 92% to 96% as measured using an internal DNA standard. Based on the high recovery and quantitative outcome, the optimized protocol provides a starting point for subsequent quantitative DNA analyses. However, just as important, the optimized steps in the present protocol, we believe, provide a guideline for most studies based on DNA extracted from seawater samples.

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Submitted 25 November 2003
Revised 29 March 2004
Accepted 22 August 2004