APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2008, p. 4336–4345 Vol. 74, No. 14
0099-2240/08/$08.00+0 doi:10.1128/AEM.02131-07

Development of a Denaturing High-Performance Liquid Chromatography Method for Detection of Protist Parasites of Metazoans▽†

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Received 17 September 2007/Accepted 12 May 2008

Increasingly, diseases of marine organisms are recognized as significant biotic factors affecting ecosystem health. However, the responsible disease agents are often unknown and the discovery and description of novel parasites most often rely on morphological descriptions made by highly trained specialists. Here, we describe a new approach for parasite discovery, utilizing denaturing high-performance liquid chromatography (DHPLC) reverse-phase ion-paring technology. Systematic investigations of major DHPLC variables, including temperature, gradient conditions, and target amplicon characteristics were conducted to develop a mechanistic understanding of DNA fragment separation by DHPLC. As a model system, 18S rRNA genes from the blue crab (*Callinectes sapidus*) and a parasitic dinoflagellate *Hematodinium* sp. were used. Binding of 18S rRNA gene PCR amplicons to the DNA separation column in the presence of triethylammonium acetate (TEAA) was inversely correlated with temperature and could be predicted based on the estimated DNA helicity of the PCR amplicon. Amplicons of up to 498 bp were resolved as single chromatographic peaks if they had high (>95%) DNA helicity. Amplicons that differed by as few as 2 bp could be resolved. Separation of 18S rRNA gene PCR amplicons was optimized by simultaneous manipulation of both temperature and solvent gradients. The optimal conditions included targeting regions of high DNA helicity (>95%), temperatures in the range of 57 to 63°C, and a linear acetonitrile gradient from 13.75 to 17.5% acetonitrile in 0.1 M TEAA (55 to 70% buffer B) over a 9-min period. Under these conditions, amplicons from a variety of parasites and their hosts can be separated and detected by DHPLC.

Denaturing high-performance liquid chromatography (DHPLC) is a relatively new analytical technique that has been used primarily in human clinical medicine to detect variant PCR-amplified genes and disease mutations in biological samples containing mixtures of homologous genes (26–28, 37, 39). More recently, DHPLC has begun to find a wider application in the fields of clinical and environmental microbiology, immunology, virology, and toxicology. An exciting new DHPLC application is its use for separating and identifying bacterial, fungal, and microeukaryotic genes from mixed samples. For example, DHPLC has been used to identify different 16S rRNA genes present in mixed bacterial cultures (2, 3, 12) and in urine samples from human patients with polymicrobial genitourinary system infections (5, 7, 17). DHPLC has also been used for diagnosis of fungal infection by targeting the ribosomal internal transcribed spacer 2 sequence (11) and for typing of a wide variety of other microorganisms and target genes (1, 18, 38). However, despite its promise as a tool for high-throughput profiling of symbiotic communities of microeukaryotic organisms, to our knowledge there has never been a systematic study conducted to determine general rules and conditions for developing such DHPLC-based assays.

Separation of PCR amplicons by DHPLC is based on ion pair reverse-phase liquid chromatography (16). DNA amplicons are retained on a column composed of hydrophobic C_{18} polystyrene divinyl benzene beads that are positively charged with the ion-pairing agent triethylammonium acetate (TEAA). Under these conditions, the negatively charged DNA phosphate backbone is electrostatically bound to the positively charged ammonium group of TEAA. After the binding, an increasing gradient of acetonitrile is applied to the column and, depending on the strength of the ionic bonds between the DNA fragments and the charged beads, DNA fragments are released from the column, passed through UV and fluorescence detectors, and can be collected using a fraction collector. Because differences in primary structure (sequence) affect the denaturation properties of nucleic acid molecules (6), DNA fragments with sequence differences and/or size differences can theoretically be separated by DHPLC. However, relatively little is known about the practical application of DHPLC technology for separating and distinguishing amplified gene fragments, and the technology has not yet been widely applied outside human clinical medicine.

Isolation and identification of microbial species in a mix-

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 23 May 2008.

TABLE 1. Universal 18S rRNA gene primers used in this study[a]

Primer	Primer sequence (5′ to 3′)	Source or reference
Univ F-15	CTG CCA GTA GTC ATA TGC	13
Univ R-565	TTA CCG CGG CTG CTG G	This study
Univ F-907	GAG GTG AAA TTC TTR GA	This study
Univ F-1131	AAA CTY AAA GRA ATT GAC GG	This study
Univ R-1428	CTA AGG GCA TCA CAG ACC	This study
Univ R-1629	GAC GGG CGG TGT GTR C	13

[a] IUPAC degenerate bases: Y represents C or T; R represents A or G.

ture by DHPLC requires that primers used in the initial PCR amplification target a gene fragment that is divergent enough to allow phylogenetic identification based on sequence. Additionally, the primers need to target a sequence that will amplify genes from all eukaryotic organisms without bias. Therefore, the 18S small ribosomal subunit is an attractive target for investigating protist parasites and prey organisms since it contains a number of highly conserved regions shared by the large majority of eukaryotic organisms while also containing regions of higher variation (10, 31). Furthermore, PCR amplification of the 18S rRNA gene fragments is highly efficient since this gene is present as multiple copies in eukaryotic genomes.

In this study a DHPLC assay was developed to allow the separation of 18S rRNA PCR-amplified gene fragments produced from a mixture of eukaryotic species. As a model experimental system, we utilized a parasitic dinoflagellate (*Hematodinium* sp.) which infects the crustacean *Callinectes sapidus* (blue crab) (22). Using this model, we demonstrated that 18S rRNA gene amplicons of hosts and parasites could be separated and identified by DHPLC and determined the primary physical and chemical conditions that allowed optimal separation. To determine the amount of sequence variation between gene fragments that could be resolved by DHPLC separation, PCR amplicons produced from a mixed culture of the cryptophyte alga *Rhodomonas* sp. was also utilized. On the basis of these studies, we propose a general strategy for development of DHPLC assays for separations of amplicons from different organisms.

MATERIALS AND METHODS

Experimental system. To develop and optimize DHPLC separation procedures for detection and discovery of prey and parasites of crustaceans, a model system consisting of cloned 18S rRNA genes and genomic DNA from an algal culture was utilized. Near-full-length small-subunit (SSU) rRNA genes originating from the blue crab (*Callinectes sapidus*) and the parasitic dinoflagellate *Hematodinium* sp. were cloned into the bacterial plasmid vector pCR 4-TOPO (Invitrogen) as previously described (13) and used as model genes for DHPLC separation method development studies. Plasmid DNA was purified from the pCR 4-TOPO vector by using a High Pure plasmid isolation kit (Roche), following the manufacturer's instructions. Genomic DNA purified from the cryptophyte alga *Rhodomonas* sp. was also examined by DHPLC. *Rhodomonas* sp. cultures originally identified as *Rhodomonas baltica* were obtained from IFREMER, Brest, France, and cultured in f/2 medium diluted 10× (f/20) (13a) with a 14:10-h light cycle at 15°C as previously described (25). Genomic DNA from algal cultures was purified using Qiagen's DNeasy blood and tissue kit (Qiagen, Inc.) as recommended by the manufacturer.

Universal eukaryotic primer design and PCR optimization. Six 18S rRNA gene PCR primers were designed and utilized in this study (Tables 1 and 2). Each of these primers targeted the most highly conserved regions of the 18S rRNA gene and is therefore referred to as a "universal" eukaryotic primer. Each primer set amplified fragments of the 18S rRNA gene ranging in size from 297 bp to 722 bp. Four primer sets amplified fragments in the 3′ region of the molecule, and one primer set amplified a 550-bp fragment near the 5′ end of the molecule (see Fig. S1 in the supplemental material). To facilitate primer design, a representative database of 102 aligned complete and nearly complete 18S rRNA gene sequences representative of most eukaryotic lineages, with the exception of true fungi and oomycetes, was assembled in the sequence editor database BioEdit version 7.0.5.3 (14). Conserved regions were identified using available utilities in BioEdit, and primers targeted to these regions were designed using the primer design software packages Primer Premier version 5.00 (Premier Biosoft International), GeneTool Lite version 1.0 (BioTools, Inc.), and Primer3 (http://fokker.wi.mit.edu/primer3/). Primer universality was assessed in silico using the Probe Match utility in Ribosomal Database Project version 8.1, which allows searches against an unaligned eukaryotic SSU RNA database (http://rdp.cme.msu.edu/), and using the BLAST utility for short, nearly exact sequences within the nucleotide (nr/nt) collection (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Primer pairs were optimized for PCR by using Optimase DNA polymerase (Transgenomic, Omaha, NE). This high-fidelity, thermal-stable DNA polymerase produces blunt-end amplicons and is recommended for use with DHPLC analyses (23). PCR conditions were optimized to yield single amplicons of the expected size with minimal primer dimer formation. Optimal annealing temperature, cycle number, Mg^{2+}, and DNA template concentrations were determined for each primer pair. Optimal annealing temperatures for each primer pair used in this study are shown in Table 2. All other PCR assay conditions were standardized and included template concentration of 10^8 copies of the target gene for plasmids or 5 to 30 ng genomic DNA, 2 mM Mg^{2+}, ca. 0.2 μM (1 ng/μl) of each primer, and 0.2 mM each deoxynucleoside triphosphate per 20-μl reaction mixture. The PCR amplification conditions included an initial denaturation cycle (5 min at 95°C), followed by 25 amplification cycles (30 s at 95°C, 30 s at the annealing temperature [Table 2], and 1 min and 72°C). The amplification cycles were followed by a final extension at 72°C for 5 min. PCR was performed using a GeneAMP 9700 thermal cycler (Applied Biosystems, Foster City, CA). Template DNA concentration was estimated by fluorometry, using a Turner Design TD-700 fluorometer (Turner Designs, Sunnyvale, CA) after staining with PicoGreen (Molecular Probes, Eugene, OR). Amplified gene fragments were visualized and sized by agarose gel electrophoresis in 1% gels stained with GelStar nucleic acid stain (Cambrex). Only combinations of forward and reverse primers that specifically amplified the expected size products from the three primary model species (*Callinectes sapidus*, the *Hematodinium* sp., and the *Rhodomonas* sp.) were used in these studies.

DHPLC optimization and investigation of factors influencing separation of 18S rRNA gene fragments. PCR amplicons were injected onto a Transgenomic WAVE DHPLC equipped with a DNASep HT cartridge (Transgenomic, Omaha, NE; catalog no. DNA-99-3710). No additional purification of PCR products was required for DHPLC separation. Several chemical and physical parameters were evaluated to optimize separation of 18S rRNA gene fragments,

TABLE 2. Optimal DHPLC conditions for PCR primer sets used in this study[a]

Forward primer	Reverse primer	Product length (bp)	PCR annealing temp (°C)	DHPLC temp (°C)	Gradient (% buffer B)	Gradient slope (% ACN/min)
Univ F-1131	Univ R-1428	297	57.4	59.5	60–79	0.28
Univ F-907	Univ R-1428	521	55.0	61.5	55–70	0.42
Univ F-15	Univ R-565	550	50.0	57.0	65–70	0.14
Univ F-907	Univ R-1629	722	50.0	63.0	60–70	0.28
Univ F-1131	Univ R-1629	498	50.0	59.5	60–70	0.28

[a] Amplicon target location is shown in Fig. S1 in the supplemental material. ACN, acetonitrile.

including column (oven) temperature, elution gradient conditions, and flow rate. Linear elution gradients were formed with WAVE optimized buffer A, consisting of 0.1 M TEAA, and WAVE optimized buffer B, consisting of 0.1 M TEAA in 25% (vol/vol) acetonitrile. Separated PCR amplicons were detected by UV (260 nm), using a UV detector (L-7485), and more sensitively by fluorescence, using an HSX-3500 fluorescence detector (excitation at 490 nm, emission at 520 nm), after staining the DNA fragments in the DHPLC flow path with either WAVE optimized HS staining solution I (Transgenomic; catalog no. 553440) or a solution of Sybr gold (Molecular Probes, Inc.) (excitation at 495 nm, emission at 537 nm). A working concentration of Sybr gold was achieved by diluting the stock solution 1:12,500 in MilliQ water. Staining with Sybr gold yielded equivalent results compared with the proprietary HS staining solution and was considerably less expensive (data not shown). Analytical grade buffers and solutions, with the exception of Sybr gold, were purchased from Transgenomic (Omaha, NE). Analysis of DHPLC results (chromatographs) was facilitated using Navigator software version 1.6.2 (build 12) (Transgenomic, Omaha, NE).

The optimal conditions for separation of PCR amplicons were determined by investigating the effect of loading concentration, column temperature, elution gradient conditions, and column flow rate. Initial studies indicated little effect of PCR amplicon loading concentrations between 25 and 100 ng in a 5-μl injection, so for all studies, 25 ng was injected onto the DNASep column (data not shown). The best separation using a linear gradient was achieved at the lowest flow rates (data not shown), so the lowest flow rate tested (0.35 ml min^{-1}) was used in all subsequent studies. The effects of column temperature and gradient slope on fragment separation were investigated by varying both parameters systematically. Column temperature was varied from 57 to 65°C. Linear gradients were varied from starting concentrations of 35% buffer B (65% buffer A) to 65% buffer B (35% buffer A) and a fixed ending concentration of 70% buffer B (30% buffer A). The optimal conditions were identified by maximizing the parameter (γ) for each chromatographic peak ($\gamma = \Delta \times \alpha$), where Δ is the distance between the closest peak (minutes) and α is the peak area (optical density units). The overall best conditions for separation of several peaks were identified by maximizing the product of each γ ($\Pi_\gamma = \gamma_1 \times \gamma_2 \ldots \gamma_i$). In this study, where we separated 18S rRNA gene fragments from *Callinectes sapidus* and the *Hematodinium* sp., optimization was achieved by maximizing $\Pi_\gamma = \gamma_{Hematodinium\ sp.} \times \gamma_{C.\ sapidus}$.

Effect of amplicon size and sequence on DHPLC separation performance. To determine the influence of PCR amplicon fragment length and sequence on DHPLC separation performance, two sets of studies were conducted. First, different regions of the 18S rRNA gene from the blue crab (*C. sapidus*) plasmid clone were amplified using the primer pairs shown in Table 2. These amplicons were further separated under previously determined optimal and nonoptimal temperature and gradient conditions. In a second set of studies, the 498-bp PCR amplicon produced from the primer pair consisting of Univ F-1131 and Univ R-1629 was used to amplify genomic DNA from the *Rhodomonas* sp. algal culture. Since cryptophytes contain nucleomorphic ribosomal genes (20), it was expected that these primers would amplify both the 18S rRNA gene and the nucleomorph gene. The separation of fragments produced in these experiments was correlated with a number of parameters, including fragment size, location, GC content, and predicted DNA helicity. Estimation of fragment helicity was facilitated as originally defined by Fixman and Freire (6) and implemented in Navigator software version 1.6.2 (build 12) (Transgenomic, Omaha, NE).

Collection and sequence analysis of DHPLC-separated amplicons. To confirm and/or identify DHPLC chromatographic peaks by sequencing, peaks were collected using an FCW-200 fraction collector (Transgenomic, Omaha, NE) integrated with the WAVE DHPLC system, reamplified with the primers used to produce the original amplicon, cloned into a sequencing plasmid vector, and sequenced. Reamplification was facilitated using a HotMaster *Taq* DNA polymerase kit (Eppendorf, North America, Inc.), and amplicons were gel purified using Quantum Prep Freeze 'n Squeeze DNA gel extraction spin columns (Bio-Rad Laboratories, Inc.) and ligated into a pCR4-TOPO vector by using a TOPO TA cloning kit (Invitrogen Corp.) according to the manufacturer's instructions. For sequencing, plasmids were purified using a High Pure plasmid isolation kit (Roche), sequenced by capillary electrophoresis using a CEQ DTCS quick-start sequencing kit, and analyzed using a CEQ 8000 eight-channel capillary sequencer (Beckmann Coulter, Inc.). The identities of the fragments were initially determined by comparison to sequences in GenBank, using the BLASTn utility to search the nr/nt database (http://www.ncbi.nlm.nih.gov/). In some cases, it was possible to sequence directly from the reamplified PCR product without subcloning, but in all cases in this study, fragments were cloned prior to sequencing.

RESULTS

Temperature and gradient effects on single amplicons. Increasing column temperatures from 57°C to 65°C with a fixed acetonitrile elution gradient (55 to 70% buffer B) decreased the retention times of PCR amplicons (Fig. 1). Above 59°C, fragment retention time increased proportionally ($r^2 = 0.99$) with temperature. However, increasing temperature from 57°C to 59°C had little effect on DNA fragment retention time. Similarly, above 59°C eluted fragment concentration as estimated by fluorescence decreased proportionally with column temperature ($r^2 = 0.99$). Reciprocally, above 59°C the amount of DNA released in the column void volume increased with column temperature ($r^2 = 0.88$). There was no difference in either the fragment concentration or the amount of DNA present in the void volume between 57°C and 59°C. These results suggest that at temperatures below 59°C all of the PCR amplicon was bound to the column, that above 63°C none of the amplicon was bound to the column, and that between 59°C and 63°C only a portion of the PCR amplicon was bound. Thus, this was the temperature window most suitable for separation of these fragments.

The elution of a single 498-bp amplicon produced from cloned *Hematodinium* sp. and *C. sapidus* 18S rRNA genes, where the acetonitrile gradient slope was varied and the column temperature was fixed at 59.5°C, is shown in Fig. 2. In all cases, each amplicon was eluted at a constant acetonitrile concentration. The *Hematodinium* sp. amplicon eluted at 63.3% ± 0.1% buffer B (15.8% acetonitrile), and the *C. sapidus* amplicon eluted at a slightly higher acetonitrile concentration (64.9% ± 0.2% buffer B, 16.3% acetonitrile). Therefore, the elution time could be manipulated by adjusting the slope of the acetonitrile gradient. Peak retention times for both the *Hematodinium* sp. and the *C. sapidus* amplicons decreased with an increasing acetonitrile gradient slope ($r^2 = 0.99$) (Fig. 2C). Unlike variation of column temperature, variation of the gradient elution conditions used did not appear to affect the retention efficiency of the 498-bp 18S rRNA gene PCR amplicon (Fig. 2).

Because both the binding and the elution of PCR amplicons could be manipulated by column temperature and elution gradients, it was necessary to conduct experiments where both temperature and gradient conditions were simultaneously and systematically manipulated. To accomplish this, a 5-by-7 factorial experimental design between temperature and percent buffer B, respectively, in which both the elution gradient and the column temperature were varied, was utilized (Fig. 3). The elution gradient conditions were adjusted from a starting concentration of 35 to 65% buffer B (8.75 to 16.25% acetonitrile in 0.1 M TEAA) and a fixed ending concentration of 70% buffer B (17.5% acetonitrile in 0.1 M TEAA), and column temperatures were adjusted from 35°C to 65°C in 5°C increments. The optimal conditions for time separation between the *Hematodinium* sp. and the blue crab (*C. sapidus*) PCR amplicons and the peak area (amount of PCR amplicon) that was eluted within the working gradient concentration were established. This variable was defined as Π_γ (see Materials and Methods). The optimal DHPLC gradient and temperature conditions for each primer set are shown in Table 2. For each primer

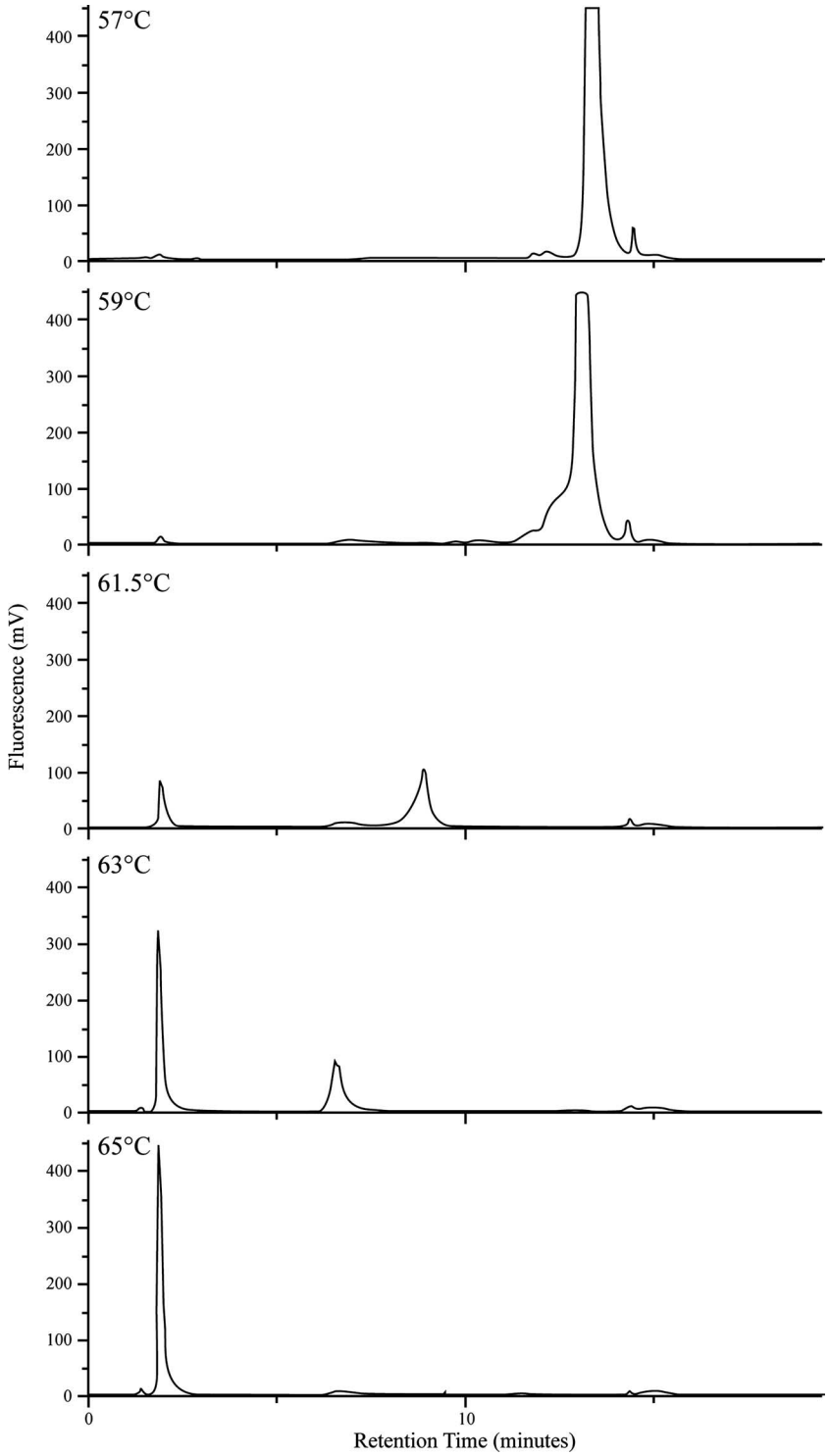

FIG. 1. Influence of temperature on DHPLC peak retention time of the 498-bp *C. sapidus* 18S rRNA gene fragment. The conditions under which the experiments were performed were a linear elution gradient of 55 to 70% buffer B (13.75 to 17.5% acetonitrile in 0.1 M TEAA) and a 0.35-ml/min flow rate. The experimental temperatures are given for each chromatograph.

set, unique conditions were required to optimize DHPLC assays that were not readily predicted based on theoretical considerations. However, the best separation was generally achieved for the different amplicons at temperatures between 57 and 63°C and relatively steep acetonitrile gradients with starting concentrations of buffer B ranging from 60 to 65% (Table 2). However, in the case of the 521-bp amplicon, a shallower gradient generated optimal conditions.

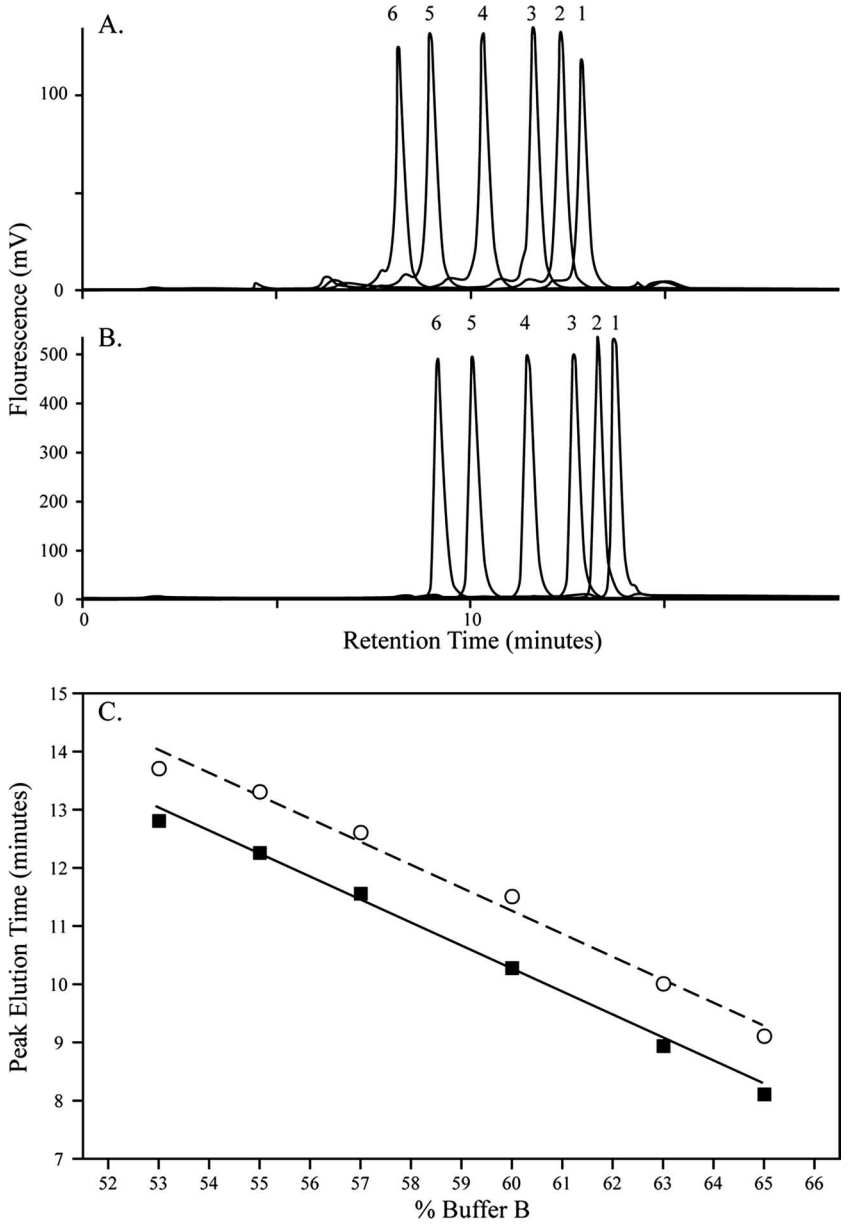

FIG. 2. Influence of acetonitrile elution gradient on DHPLC peak retention time on the 498-bp 18S rRNA gene amplicon from the *Hematodinium* sp. (A) and *C. sapidus* (B). The gradient slope was adjusted by varying the starting concentration of buffer B from 53 to 65% (13.25 to 16.25% acetonitrile in 0.1 M TEAA), with a fixed end point of 70% buffer B (17.5% acetonitrile in 0.1 M TEAA). The initial concentrations of buffer B were as follows: for peaks labeled no. 1, 53% (slope = 1.9); no. 2, 55% (slope = 1.7); no. 3, 57% (slope = 1.4); no. 4, 60% (slope = 1.1); no. 5, 63% (slope = 0.8); and no. 6, 65% (slope = 0.6). The relationship between peak retention time and initial concentration of buffer B is shown in panel C. Open circles represent *C. sapidus* and filled squares the *Hematodinium* sp.

Predicting DHPLC fragment behavior. To gain a more complete understanding of the DNA fragment properties that would contribute to its behavior on the DHPLC column, we investigated the behavior of each PCR amplicon under optimal and nonoptimal conditions and analyzed it with respect to fragment size, location within the 18S rRNA gene, average GC content, and predicted DNA helicity. These properties for each 18S rRNA gene fragment used in this study are shown in Table 3. In Fig. 4, the behavior of the five amplicons from this study under fixed DHPLC conditions of 59.5°C and 60 to 70% buffer B is shown. These conditions were found to be optimal for the 297-bp and 498-bp fragments and suboptimal for the 521-bp, 550-bp, and 722-bp amplicons (Table 2). Under these conditions, the 297-bp and 498-bp fragments from the *Hematodinium* sp. both produced single chromatographic peaks that eluted relatively late in the gradient (10.27 and 11.50 min, respectively) while the other fragments produced multiple peaks that tended to elute early in the gradient (Fig. 4). Apparent peaks at 2 to 3 min and after 15 min are nonspecific products released in the column void volume and column wash, respectively.

Fragment size did not appear to be the primary charac-

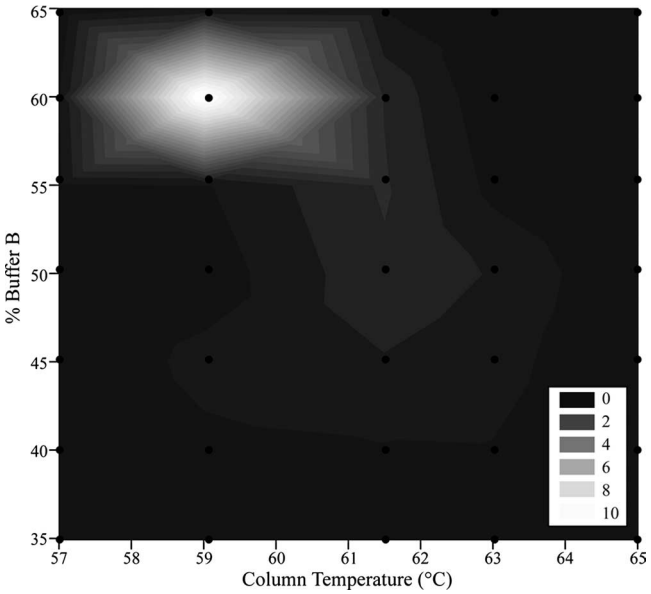

FIG. 3. Optimization of DHPLC acetonitrile elution gradient and temperature conditions. Results of a 5-by-7 factorial matrix study with mixtures of the amplified 498-bp 18S rRNA gene fragment from the *Hematodinium* sp. and *C. sapidus*. The calculated parameter II_γ ($\gamma_{Hematodinim\ sp.} \times \gamma_{C.\ sapidus}$) is shown ($10^{13}$). Symbols (●) indicate the experimental conditions tested. The optimal conditions for the detection and separation of this amplicon from these two species were a temperature of 59.5°C and an acetonitrile elution gradient from 60 to 70% buffer B (15 to 17.5% acetonitrile in 0.1 M TEAA).

teristic explaining this behavior, since two of the fragments in the range of 500 bp (521 bp and 550 bp) produced multiple peaks while the similar-sized 498-bp fragment produced a single peak. Both fragments that were resolved to a single chromatographic peak (i.e., 498 bp and 297 bp) were located in an overlapping region near the 3′ end of the 18S rRNA gene molecule, suggesting that location is an important predictive parameter. However, two other fragments in the same general region of the molecule eluted as multiple peaks, suggesting that location alone is insufficient to predict DHPLC behavior. Likewise, GC content alone did not fully explain the DHPLC behavior of these amplicons. The average GC content levels of the 550-bp and 498-bp fragments ranged from 49.7% to 53.2%, respectively, and although there was a general tendency for fragments with higher GC contents to resolve into a single DHPLC peak, the 722-bp fragment with the second highest GC content (52.4%) was not resolved in a single peak under these DHPLC conditions. Predicted DNA helicity, as estimated using the Navigator software, correlated most closely with the behavior of amplicons. At temperatures where predicted DNA helicity was above 95%, DNA fragments eluted from the DHPLC column as a single peak, whereas multiple peaks were observed when predicted helicity was less than 95%. For example, at 59.5°C the two fragments that produced a single DHPLC peak each had a predicted helicity near 100% (average = 96.6%), while at this temperature the predicted helicities for the other three fragments were lower (average = 79.7%) (Table 3; also see Fig. S2 in the supplemental material). However, in all cases, when column temperatures of 57°C were evaluated (when the predicted helicities of all fragments were greater than 95%), amplicons produced from the *Hematodinium* sp. and *C. sapidus* were found to coelute as a single peak and could not be separated (data not shown).

Distinguishing amplicons from different species by sequence. To determine whether our target amplicons from different species could be resolved by DHPLC, two experiments were conducted utilizing the 498-bp amplicon. The 498-bp amplicon was used because the predicted helicity was high and the amplicon length sufficient to allow phylogenetic identification based on sequence similarity searches. In the first study, mixtures produced from the blue crab and from *Hematodinium* sp. plasmids were loaded in a mixture onto the DHPLC column at a 1:1 ratio. In the second study, amplification of genomic DNA from a culture of the cryptophyte alga *Rhodomonas* sp. was examined after DHPLC analysis. In both experiments, multiple resolved peaks were observed (Fig. 5). DHPLC resolution of the blue crab and the *Hematodinium* sp. is shown in Fig. 5A. The 498-bp amplicon produced from the *Hematodinium* sp. eluted first at 10.27 min, followed by the blue crab at 11.50 min. The identities of both peaks were confirmed by sequencing and shared 85% nucleotide sequence similarity. The chromatograph from the genomic DNA of the algal culture was more complex, with seven resolved peaks apparent (Fig. 5B). The identity of each peak was determined by sequencing. The first two peaks (7.67 and 8.50 min) were both identified as 18S rRNA genes from the *Rhodomonas* sp. with closest identity to *Rhodomonas baltica* and *Rhodomonas maculata*. Sequence identity over the 498-bp amplicon between these species was nearly 100%, with only two nucleotide difference between them. The third (10.67 min) and fifth (13.00 min) peaks were both identified as *Rhodomonas* ribosomal nucleomorph genes that shared only 14% nucleotide identity with each other and 17% and 15% nucleotide identity with the true SSU rRNA genes. Interestingly, the fourth chromatographic peak (11.67 min) was identified as an 18S rRNA gene most closely related to the aquatic mite *Acariformes* sp., sug-

TABLE 3. Amplicon properties

Product length (bp)	Location (5′–3′)	% GC content	Avg % DNA helicity at:			
			57.0°C	59.5°C	61.5°C	63.0°C
297	1131–1428	51.1	99.9	97.7	2×10^{-2}	8×10^{-4}
521	907–1428	50.7	99.6	74.0	4×10^{-2}	2×10^{-3}
550	15–565	49.7	75.0	55.5	1.4	1×10^{-1}
722	907–1629	52.4	99.7	79.5	1.2	1×10^{-2}
498	1131–1629	53.2	99.5	96.2	1.6	1×10^{-2}

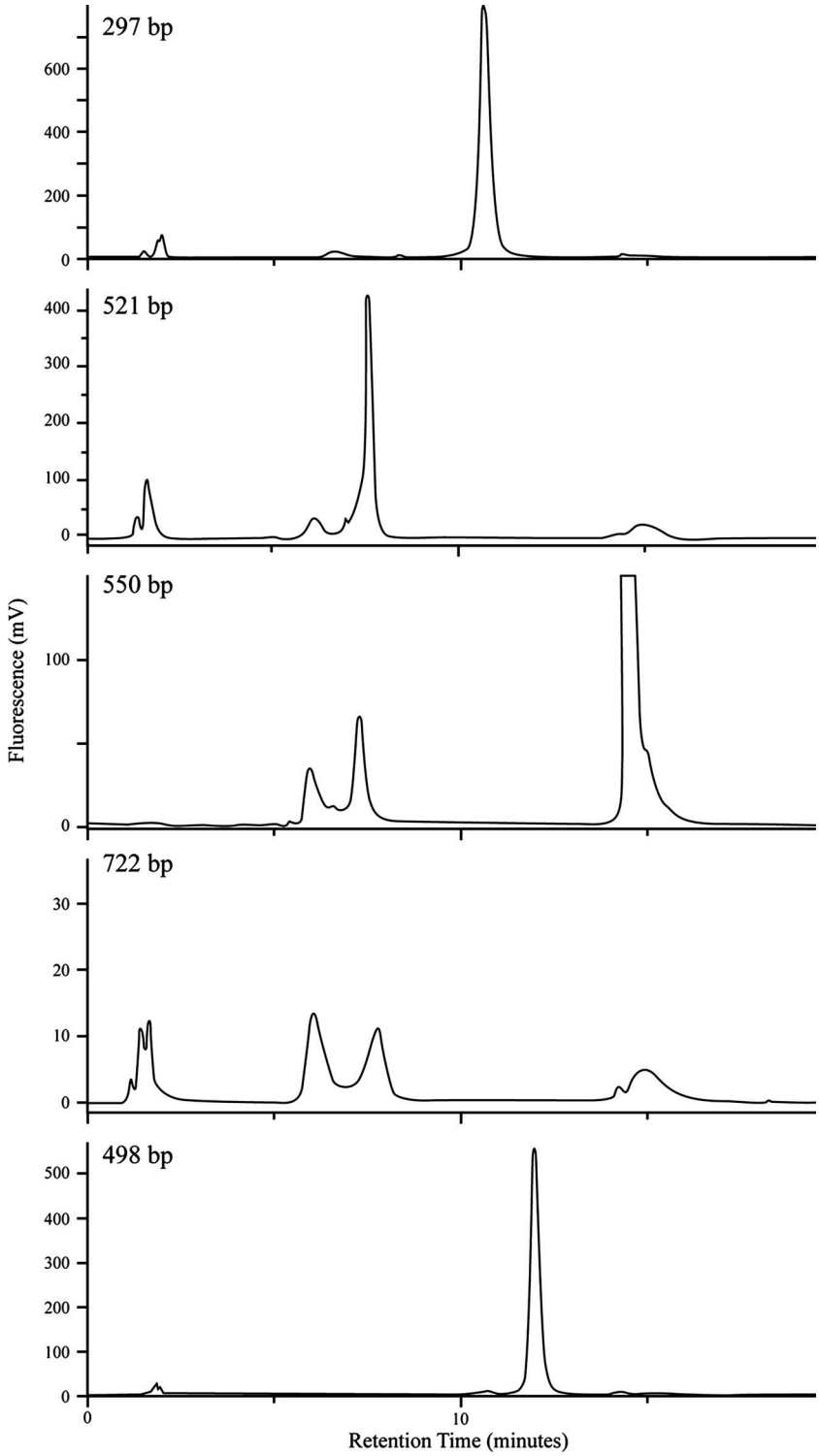

FIG. 4. DHPLC detection of 18S rRNA gene amplicons investigated in this study under optimal and nonoptimal conditions. Amplicons which were resolved are indicated in each panel. All separations shown were produced with a column temperature of 59.5°C and an elution gradient from 60 to 70% buffer B (15 to 17.5% acetonitrile in 0.1 M TEAA). Note that no quantitative analysis was preformed, only a behavioral analysis based on peak resolution and definition.

gesting that the culture was contaminated by this mite. Within this fragment, the 18S rRNA gene was only 29% similar to the *Rhodomonas* sp. 18S rRNA gene. The sixth peak (15.75 min) was not identified, and the seventh peak (18.00 min) was the column wash. Although the most similar amplicons eluted most closely to each other, there was not a significant correlation between sequence similarity and elution separation ($P = 0.153$).

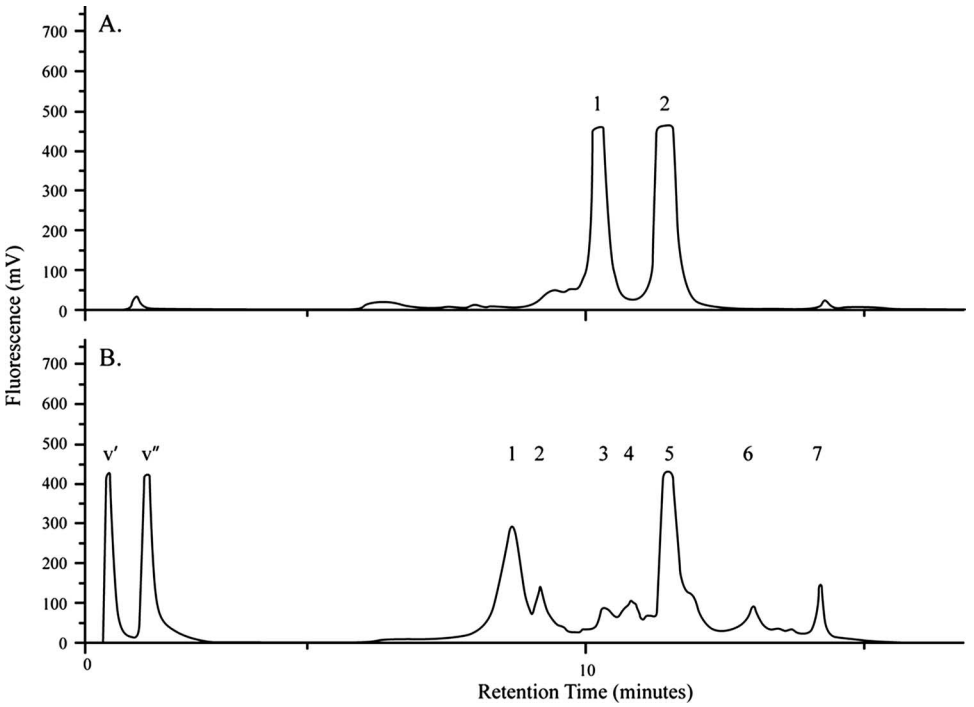

FIG. 5. DHPLC separation of DNA mixtures of the 498-bp amplicon from the *Hematodinium* sp. (A) and *C. sapidus* and a culture of the cryptophyte alga *Rhodomonas* sp. (B). Peak 1 in panel A is the *Hematodinium* sp., and peak 2 is *C. sapidus*. In panel B, peaks 1 and 2 were both identified as *Rhodomonas* sp. 18S rRNA gene amplicons, and peaks 3 and 5 were identified as ribosomal nucleomorph genes. Peak 4 was identified as most closely related to an rRNA gene from the aquatic mite *Acariformes* sp. Peak 6 could not be identified, and peak 7 is the column wash. The peaks identified as V′ and V″ are injection peaks associated with the column void volume.

DISCUSSION

PCR-based diagnostic approaches have become widely used in a variety of clinical and ecological studies to detect, identify, and describe microorganisms (29, 33). Most recently, DHPLC, originally developed to detect single nucleotide polymorphisms and gene mutations (39), has been applied in a partially denaturing mode to separate mixtures of PCR amplicons derived from different organisms (2–5, 11, 12). Here, we report the first evaluation and the development of a DHPLC approach targeting 18S rRNA gene fragments for the detection and discovery of parasites in metazoan hosts. This study focused on developing proof of concept and determining a practical approach for designing DHPLC assays targeting the 18S rRNA gene. As a model system, we chose to use as a model host and parasite system the blue crab (*C. sapidus*) and the parasitic dinoflagellate *Hematodinium* sp. because a *Hematodinium*-specific PCR assay was available for direct comparison with DHPLC results. In a second companion study, we evaluated, developed, and increased the sensitivity of the DHPLC assay for successful detection and identification of parasites in the hemolymph of wild-caught *C. sapidus*, overcoming the problem of PCR template bias (30) occurring when host DNA is greatly more abundant then parasite DNA (36). Infections of the *Hematodinium* sp. are thought to have dramatically reduced populations of the commercially important blue crab along the eastern U.S. coast (8, 21, 22, 32).

The goal of our studies was to develop a strategy and an assay for separating mixtures of 18S rRNA gene amplicons from different organisms. Factorial design studies utilizing known mixtures of 18S rRNA gene amplicons covarying temperature and gradient conditions allowed the identification of optimal separation conditions for a given PCR amplicon. Optimal conditions were achieved by maximizing peak separation (Δ) and peak area (α). Peak separation was largely a function of gradient conditions, while peak area was affected primarily by temperature.

The theoretical basis of the behavior of partially denatured double-stranded-DNA fragments with different primary structure during DHPLC is discussed in several recent reviews. Xiao and Oefner (39) noted that at partially denaturing temperatures the behavior of DNA fragments on the DHPLC column is dictated by the content and distribution of AT base pairs. It has been hypothesized that amplicons with decreased retention times had low-melting-temperature domains and were therefore partially denatured at lower acetonitrile concentrations (15, 27). Our studies, in agreement with previous reports, found a general correspondence between the GC content of a gene fragment and its behavior on the DHPLC column. However, a better predictor of amplicon DHPLC behavior, particularly the ability to resolve a specific fragment into a single peak, was the predicted helicity of the amplicon at the assay temperature. Amplicons with predicted helical fractions of greater than 95% produced single peaks, while fragments with lower predicted helical fractions generated multiple peaks. Similarly, other parameters reported elsewhere to effect DHPLC behavior, including fragment size and predicted melting temperature, were not reliable predictors of the behavior of 18S rRNA gene amplicons in these studies. Based on our

TABLE 4. DHPLC assay design strategy for Wave DHPLC systems equipped with a DNASep HT cartridge

Step no.	Description
1	Identify target region and design suitable PCR primers by using standard PCR primer design criteria[a]. Optimal target regions should exhibit >95% avg helicity and be large enough[b] to enable phylogenetic identification based on sequencing.
2	Develop model exptl system with cloned target genes consisting of at least two target species.
3	Empirically evaluate DHPLC conditions by utilizing a factorial exptl design. Temp should be varied from 53 to 65°C and gradient conditions from 35 to 65% starting concn of buffer B (8.75 to 16.25% acetonitrile), with an ending concn of 70% buffer B (17.5% acetonitrile).
4	Mobile phase flow rates can be adjusted to minimize run conditions and reagent use.

[a] Goldenberg et al. (12) recommend the use of GC clamps to improve amplicon stability.
[b] Fragments up to 500 bp appear to be suitable, although larger fragments may also be useful if they conform to helicity requirements.

results, we present a generalized outline for developing DHPLC assays suitable for separating and identifying mixtures of PCR-amplified DNA fragments (Table 4).

An important question of relevance to the resolution of mixed 18S rRNA gene amplicons by DHPLC is the relationship between phylogenetic relatedness and separation. Ideally, rRNA genes from closely related organisms would elute with similar retention times, while amplicons from more distantly related species would be expected to be more widely separated. This generally appeared to be the case, but there was not a direct correspondence between sequence similarity and retention times. As with other techniques, such as denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis, which take advantage of the denaturing properties of DNA molecules, separation depends on the presence of melting domains rather than direct sequence similarity (19, 24). A significant advantage of DHPLC over these gel-based separation technologies is that DHPLC is consistently more reproducible, simpler, faster, and cheaper. However, as with denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis, it is possible that amplicons with different sequences may coelute if their melting properties are similar. Nonetheless, our results demonstrate that it is possible to separate 18S rRNA gene amplicons that differed by as few as 2 base pairs. Further, we have separated polymorphic regions of the 498-bp 18S rRNA gene fragment from the copepod species *Calanus finmarchicus* differing by as few as a single base pair (data not shown).

Within the broadly defined field of molecular ecology, the use of molecular methods and specific genetic markers is becoming an important approach to investigating the interactions between organisms (25, 34). This is especially true for studying interactions between macro- and microscopic organisms, where traditional morphological and biochemical techniques often do not provide sufficient resolution or are exceedingly laborious. Two such examples include investigations of parasitism (8) and trophic interactions in zooplankton (9, 25, 35). Here, we have shown that 18S rRNA gene amplicons can be easily separated and identified by DHPLC analysis and propose a general approach for the design of DHPLC assays. It is likely that this technique will provide a powerful new tool for investigation of the interactions between organisms, augmenting the more traditional approaches of histology and biochemical analyses.

ACKNOWLEDGMENTS

This work was supported in part by the U.S. Department of Energy Biotechnology Investigations/Ocean Margins Program (FG02-98EF-62531), the NOAA National Sea Grant College Marine Environmental Biotechnology Program (NA 06RG0029), and the U.S. National Science Foundation Office of Polar Programs (OPP-00-83381). C. Troedsson was supported by the Department of Biology at the University of Bergen. P. Simonelli was supported by the Department of Biology at the University of Bergen and by L. Meltzer Høyskolefond.

We thank Anna Boyette, Skidaway Institute of Oceanography, who prepared the figures; Oliver Goldenberg for useful discussions; and five anonymous reviewers for useful comments and criticism that substantially improved the manuscript.

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