

**Energy Research and Development Division
FINAL PROJECT REPORT**

**EVALUATION OF DNA BARCODING
AND QUANTITATIVE PCR FOR
IDENTIFICATION AND
ENUMERATION OF INVERTEBRATE
LARVAE ENTRAINED BY ONCE-
THROUGH SEAWATER COOLING
SYSTEMS**

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PREFACE

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Evaluation of DNA barcoding and quantitative PCR for identification and enumeration of invertebrate larvae entrained by once-through seawater cooling systems is the final report for the Environmental Effects of Cooling Water Intake Structures project (Contract Number 500-04-025) conducted by Moss Landing Marine Laboratories. The information from this project contributes to Energy Research and Development Division's Energy-Related Environmental Research Program.

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ABSTRACT

One difficulty of assessing the impacts of electric power plant once-through cooling systems is identifying and enumerating the larvae of organisms living on, in, or near the bottom of water bodies that may become entrained and killed. The research reported here addressed two aspects of molecular analysis of plankton important for developing the tools and protocols for efficient and high-throughput plankton analysis. The first was the application of the deoxyribonucleic acid barcoding concept to plankton studies. The goals were to test the efficacy of barcoding for identifying larvae and building a database of DNA sequence and photographs from larvae drawn from Elkhorn Slough. The second goal was to explore quantitative polymerase chain reaction as a means of enumerating larvae in samples.

Approximately 1,100 planktonic organisms from 12 plankton samples were photographed and used for deoxyribonucleic acid extraction. Information on the majority of these specimens was entered into a web-accessible database. The majority of organisms sampled and sequenced were annelid, molluscan, and crustacean, but only one could be identified at a species level by querying Genbank, a genetic sequence database. The average match of metazoan sequences to GenBank records was 16 percent, which suggested that genus or family level of identification was typical. This highlighted the fact that the Californian estuarine biota is poorly represented in existing genetic databases. Two key recommendations are to develop molecular methods for plankton characterization from unsorted plankton, and to barcode identified adult samples to build a database adequate for matching larvae and adults from deoxyribonucleic acid sequences. The authors concluded that quantitative polymerase chain reaction can be applied to real-world analysis of focal organisms, and that *Artemia nauplii* (being absent from most aquatic environments) can be routinely added to plankton samples to serve as an internal control for all steps in the procedure.

Keywords: DNA Barcoding, Quantitative PCR, Plankton, Larval Identification

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EXECUTIVE SUMMARY

Introduction

A challenge for determining the impacts of electric power plant once-through cooling systems is the inability to identify and enumerate the larvae of organisms that may become entrained and killed. This is particularly true for the larvae of invertebrates living on, in, or near the bottom of water bodies, which are very small and not typically identifiable using microscopic techniques currently employed. This study investigated the potential for deoxyribonucleic acid (DNA) barcoding and quantitative polymerase chain reaction (QPCR) for identifying such larvae. Genetic markers for identification of meroplankton have attractive qualities. First, genetic markers do not change during the lifecycle, unlike morphology, so they can be applied to meroplankton in any stage of development. Second, molecular methods for detecting genetic markers can be automated, which may allow high-throughput protocols for plankton analysis.

Project Purpose

The research reported here addressed two aspects of molecular analysis of plankton important for developing the tools and protocols for efficient and high-throughput plankton analysis. The first was applying the “DNA barcoding” concept to plankton studies. The goals were to test the efficacy of barcoding for identifying larvae and building a database of DNA sequence and photographs from larvae drawn from Elkhorn Slough. The second goal was to explore quantitative polymerase chain reaction (QPCR) as a means for enumerating larvae in samples. The researchers used larvae of the brine shrimp *Artemia franciscanus* as a model organism that could be added to natural plankton samples in known numbers, or as DNA spiked into samples at known concentrations in order to test the sensitivity of QPCR to known larval densities.

Project Results

For the DNA-barcoding study, 1101 planktonic organisms from 12 biweekly plankton tows between November 2006-May 2007 were photographed and used for DNA extraction. Information on the majority of these specimens was entered into a web-accessible database. One hundred sixty-eight DNA sequences (“barcodes”) were obtained, including controls from seawater. The majority of organisms sampled and sequenced were annelid, molluscan, and crustacean. There was no bias in barcoding success rate among taxa. The overall success rate was about 20 percent, reflecting processing inefficiencies and a high rate of return of symbiotic and seawater bacteria. The sequences were then compared to GenBank, a genetic sequence database. The average match of sequences to GenBank records was 16 percent, suggesting that genus or family level identification was typical and probably to be expected. Californian estuarine biota was poorly represented in existing genetic databases; only one larva was identified to species by comparison to Genbank.

QPCR was successful for detecting *Artemia nauplii* in DNA extractions from bulk, unsorted plankton. A single nauplius is easily detected when added to 20 milligrams (mg) of wet, packed plankton, with distinguishable separation between one and two added *Artemia nauplii*. QPCR can be used to enumerate larvae in plankton DNA diluted to a concentration of maximum

sensitivity. Background plankton DNA had no effect on the specificity of reactions, indicating that target organisms can be selectively measured without concern for variation due to differences in the background plankton community among samples. Samples also could be diluted to eliminate inhibition by competing DNA in the samples. Overall, QPCR was specific, sensitive, repeatable, and robust.

Two key recommendations are developing molecular methods for plankton characterization from unsorted plankton, and barcoding identified adult samples to build a database adequate for matching larvae and adults from DNA sequences. The researchers also concluded that QPCR could be applied to real-world analysis of focal organisms, and that *Artemia nauplii* (being absent from most aquatic environments) could be routinely added to plankton samples to serve as an internal control for all steps in the procedure.

Project Benefits

This project successfully demonstrated that DNA barcoding and quantitative polymerase chain reaction were effective techniques for determining the impacts of once-through cooling systems on larvae. The data in this report can be used by scientists, regulators, and electric utilities for evaluating cooling system technologies and Clean Water Act regulations designed to protect aquatic organisms.

CHAPTER 1:

Background

Complete assessment of the environmental impact of once-through-cooling technology associated with thermal generation requires identification and enumeration of planktonic organisms that are entrained, impinged, or otherwise disturbed in cooling systems (Strange et al. 2004, York et al. 2005). The potential magnitude of impact is great: about 17 billion gallons of seawater per day are used in once-through-cooling for energy generation in California, killing a large but unknown number of planktonic organisms (York et al. 2005). In an earlier study, we found monthly means of 5,000-90,000 unidentified bivalve larvae m⁻³ in a study at Morro Bay in 2001 (Geller and Bartl, unpublished data). If these values are used, 3.22 x 10¹¹- 2.5 x 10¹² clam larvae are killed daily in California. Predicting community impacts requires species-level identifications. However, traditional methods of plankton analysis are inadequate for this task because guides to species-level identification are few and incomplete, particularly for meroplankton, the planktonic larvae of benthic organisms (e.g., Shanks 2001). Further, throughput is low with manual identification and counting. Models that predict impacts of cooling losses on estuarine communities depend on input of reliable population data (Strange et al. 2004). Thus, there is a critical need for development of efficient, high-throughput methods of identification and enumeration of plankton.

As mentioned previously, a viable alternative is the application of tools relying on genetic information for identifying species. Ideally, samples of seawater containing raw (unsorted) plankton would be fed into a molecular analysis device that outputs species identifications and counts. At the onset of the research reported here, such technological tools are available but practical aspects of their application had not been adequately explored. The research reported here, therefore, addressed two aspects of molecular analysis of plankton. The first was the application of the "DNA barcoding" concept to plankton studies. The goals were to test the efficacy of barcoding for identification of larvae and to build a database of DNA sequence and photographs from meroplankton (larvae) drawn from Elkhorn Slough. The second goal of this project was to explore quantitative polymerase chain reaction (QPCR) as a means to enumerate larvae drawn from Morro Bay and Elkhorn Slough. However, preserved Morro Bay samples derived from an earlier project proved degraded, therefore the project focused on Elkhorn Slough waters. We used larvae of the brine shrimp *Artemia franciscanus* as a model organism that could be added to natural plankton samples in known numbers, or as DNA spiked into samples at known concentrations. *Artemia* was used to test the sensitivity of QPCR to known larval densities. This enabled us to investigate potential causes for variation in estimated larvae numbers without confounding factor of actual variation in population size. We also propose that species like *Artemia* can be added to all estuarine and marine plankton samples as an internal control for efficiency of each step in the QPCR process.

CHAPTER 2:

Part 1. DNA Barcoding of Meroplankton from Elkhorn Slough, CA

Assigning species identity to a single specimen with DNA markers is not difficult in principle (reviewed in Geller 2007). DNA sequencing of various genetic loci from a species and its close relatives can reveal species-specific sequences (markers). Once this is done, a variety of methods can be used to detect markers in an unknown specimen. Most molecular methods start with the extraction of DNA from the specimen, which can then be blotted and probed (Medeiros-Bergen et al. 1995), or more commonly amplified by polymerase chain reaction (PCR) using universal primers to produce a small fragment (amplicon) which is then sequenced, cut with restriction enzymes to reveal diagnostic fragments (e.g., Evans et al. 1998), or probed internally. Similarly, PCR primers can be carefully designed to produce differently sized amplicons that can be detected by gel electrophoresis (Hare et al. 2000, Watanabe et al. 2004, Hyde et al. 2005, Geller and Bartl, in prep, among many others). “DNA barcoding” consists of producing a DNA sequence of a genetic locus that has been chosen as a standard for a given taxon, as well as vouchering a specimen and its associated metadata (Ratnasingham and Hebert 2007). For plankton and other small organisms, vouchering is often not possible because the specimen is destroyed in the DNA extraction. Thus, we use the term “DNA barcode” in an informal fashion as applied to our data.

2.1 METHODS

2.1.1 Plankton Sampling and sorting

Twelve biweekly plankton samples were taken at one or two stations [numbers 1 (near the mouth and more marine) and 9 (near Kirby Park and more estuarine)] in Elkhorn Slough established for monitoring purposes by Sanctuary Integrated Monitoring Network (SiMoN) between November 6, 2006 and June 15, 2007. Tows were semi-quantitative using an 83 μm mesh net by boat (net diameter 0.5 m; tow duration 5 min; speed: ~ 1 knot, or 0.514 ms^{-1}). Live plankton were concentrated into 1 liter of source water and sorted immediately or kept, with aeration, overnight at 12°C .

For sorting, plankton were further concentrated into about 200 ml of source water. 1-5 ml aliquots were taken by transfer pipet and examined using a stereomicroscope. Sorters were instructed to isolate morphologically distinct meroplankton, thus the most common species were sampled quickly and most effort was spent searching for unique morphotypes. While our focus was on meroplankton, some holoplankton were taken as well. Individual plankters were isolated by Pasteur pipet and transferred to a microscope slide to be photographed with an Olympus BH2 compound microscope with a Nikon Coolpix 5400 mounted to a trinocular head. Various attempts to pen plankters in the field of view were attempted, including the use of depression slides and the construction of $3 \times 3 \text{ mm}$ wells on glass slides built of electrical tape. Plankton were photographed in source water, or in source water with methyl cellulose (to increase viscosity), MgCl_2 (to “relax” specimens) or nicotine (source water soaked in tobacco to

paralyze specimens). The general, preferred method for immobilizing and photographing plankters was to transfer live specimens individually into wells on slides and chill the slide (-20°C, approx 5 min), before viewing under the microscope. Several members of the Molecular Ecology laboratory at MLML participated in sorting (JG, JM, HH, EJ).

2.1.2 DNA extractions and PCR

DNA was extracted using GeneReleaser (Bioventures) reagent, a proprietary product that lyses cells and sequesters inhibitors to PCR. Larvae, without homogenization, were cycled in 10 ul of GeneReleaser in PCR plates through a program of temperature changes following the manufacturer's recommendation. 1-2 ul of Genereleaser was then transferred to 25 ul PCR reaction mixes. PCR consisted of 12.5 ul of Promega Green GoTaq master mix (containing 2X PCR buffer, dNTP, MgCl₂, and Taq polymerase), water, and primers (LCO1490: 5'-GGTCAA CAAATC ATAAAG ATATTGG-3' and HCO2198: 5'-TAAACT TCAGGG TGACCA AAAA ATCA-3') targeting mitochondrial Cytochrome c oxidase subunit I (Folmer et al. 1994). PCR reactions were run in a BioRad Peltier PTC 200 Thermal Cycler for 40 cycles consisting of: 94°C melting, 40s; 40°C annealing, 1 min; 72°C extension, 40s.

PCR products were purified for sequencing in a 96 well format using Whatman glass fiber Unifilter long drip plates (GF/D) following the manufacturer's protocol. PCR product was mixed with binding buffer and pulled by vacuum through the filter plates. DNA bound to the glass filters were eluted with 50 ul of TE. Cleaned PCR product was sequenced with ABI Big Dye 3.1 kits according to the manufacturer's protocol. Unincorporated dye was removed by ethanol precipitation, and sequencing reactions were resuspended in water. Sequencing reactions were then sent to the sequencing facility at the Monterey Bay Aquarium Research Institute to be analyzed on an ABI 3100 DNA sequencer or to a commercial provider, Geneway (Hayward, CA). PCR products were sequenced in both directions. GeneReleaser-only (blank) reaction controls were included in some amplification reactions to assess the probability of PCR amplification resulting from cross-well contamination or reagent contamination. Seawater-only controls, in which 2 uL of water, without a plankter, was placed on a slide and then loaded into GeneReleaser, were conducted in PCR reactions plates to assess the probable occurrence of amplification of bacteria or other organisms in Elkhorn Slough water. We used four extraction batches which had good sequence representation (25-41 sequences) to conduct an analysis of the rate of bacterial contamination, losses at the PCR or clean-up phases, and rate of likely taxon-misidentification (false sequence return) which could arise due to annotation error, cross-well transfer of specimen DNA, or contamination from the medium.

2.1.3 Sequence finishing

Sequence trace data were aligned and edited using CodonCode Aligner software (Codon Code Corporation). Forward and reverse sequences for each PCR product were selected and assembled using the Codon Code Aligner Assembly and Contig Editor. Forward and reverse sequences were assembled into a consensus sequence using default parameters to define regions with clear signal and matching nucleotide sequence. Sequence pairs that could not be assembled were rejected as unreliable unless the reason for assembly failure was no signal or

noise from one sequencing direction but non-ambiguous sequence (as assessed by the operator) from the other direction. Regions of poor readability at the ends of sequences were trimmed.

We produced a total of 163 edited sequences in this way, totaling 100,800 nucleotides. The sequence finishing process worked well. The finished data set contained only 0.01 percent calls of “N” –sites at which A, T, G, or C was not reliably distinguished. The next step was to verify that the sequence was *bona fide* COI and likely to derive from the target specimen. We used the NETBLAST implementation of BLAST (Basic Local Alignment and Search Tool (<http://www.ncbi.nlm.nih.gov/Genbank/>)) to compare the taxonomic affinity of top scoring sequences in GenBank to the known (higher) taxon of the target.

2.2 Results

From 12 plankton tows, 1101 individual larvae photographed and DNA extracted (Figure 1). We experienced considerable difficulty obtaining high quality photographs, primarily due to the movement of live plankton. MgCl₂ was helpful in arresting movement of crustacean larvae that use muscular contraction for locomotion, while this was ineffective on larvae using ciliary action. Nicotine was also sometimes helpful for Crustacea and Polychaeta, but seemed to act through neurotoxicity and induction of twitching or death. Brief exposure to -20o C incapacitated or killed larvae without distortion, which could then be photographed. Information associated with each sample was entered into the Plankton Database created for this purpose (described below). This database currently contains 985 data entries, including entries for controls (seawater and unrelated plankton samples). 911 samples are larval DNA extractions with a corresponding string of photos.

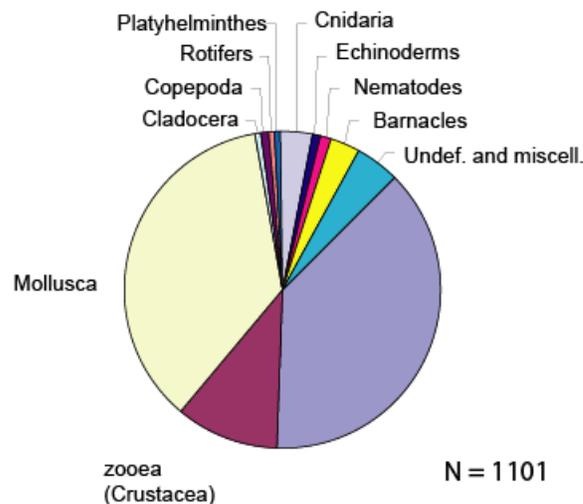


Figure 1. Diversity of higher taxa that were sorted from plankton tows in Elkhorn Slough, California between November 2006 and June 2007, photographed, and processed to extract DNA. Purple is the majority taxon, annelida.

A total of 927 PCR amplifications were attempted; time constraints did not allow the entire collection to be used. PCR was conducted in batches corresponding to individual plankton tows (equivalently, extractions plates). PCR success varied from 15.4 percent-69.6 percent, averaging 39.4 percent. Several plates showed evidence of systematic failure, indicating an unidentified problem with reagents or plankton condition prior to extractions. Excluding such, PCR success averaged 54 percent. We performed sequencing reactions on 186 PCR products (forward and reverse, or 372 reactions) using the extraction plates that did not show any such systematic problems, nor one plate that was lost in an instrument failure. 144 plankters provided readable sequence, as well as 14 sequences from seawater controls. A total of 98 sequences were consistent with the known higher taxon of the plankter, with the remainder of bacterial origin, inconsistent with the known taxon, or unassignable (Figs. 2 and 3). Many of the sequences that we cannot reliably infer to be metazoan (including many sequences in extraction batches E4 and E5) are closely related to bacterial sequences generated in a zooplankton COI Barcoding Project in Jiaozhou Bay, China (Men, R. -X., et al. unpubl. data as reported in Genbank).

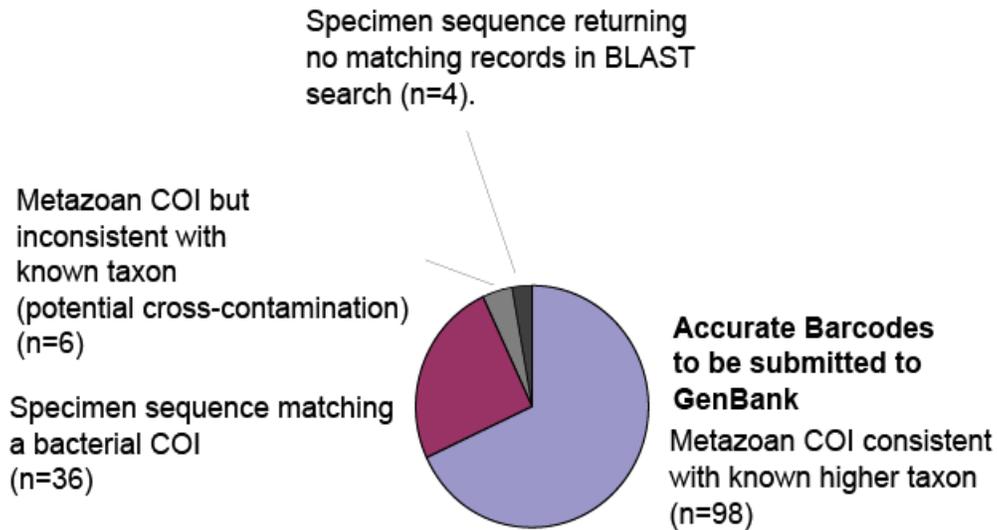


Figure 2. Distribution of sequences to categories of: metazoan COI consistent with the known higher taxon of the specimen; COI of bacterial origin, metazoan COI inconsistent with known taxon; or not definitively COI (no match in GenBank) as determined by BLAST searches of GenBank.

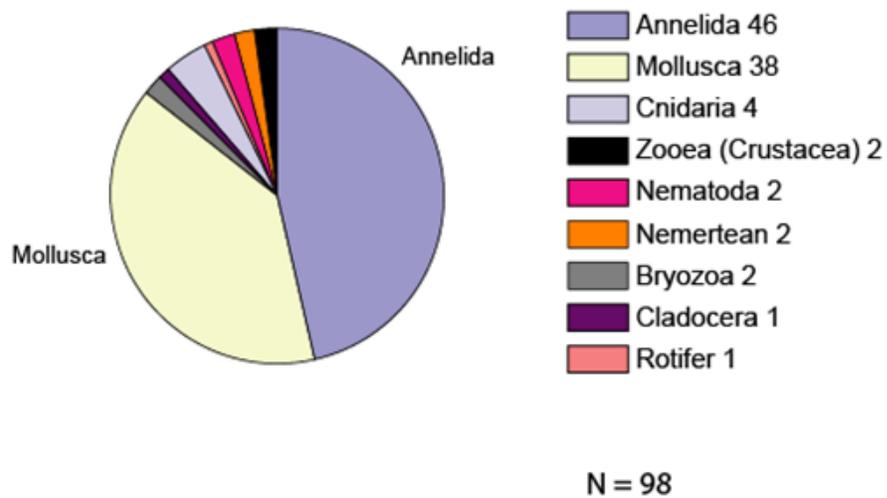


Figure 3. Diversity of taxa with verified COI (see Fig. 2).

2.2.1 Factors affecting success rate

We analyzed success rates at each step for 307 samples from four sampling dates that showed acceptable PCR success rates (>35 percent; Figure 4). Approximately 40 percent of potential barcode sample data was lost at each step of the process: PCR amplification, sequencing, and losses due to amplification and sequencing of bacteria. There was, however, no evident bias among major taxa (Annelida, Crustacea, and Mollusca) (Table 1).

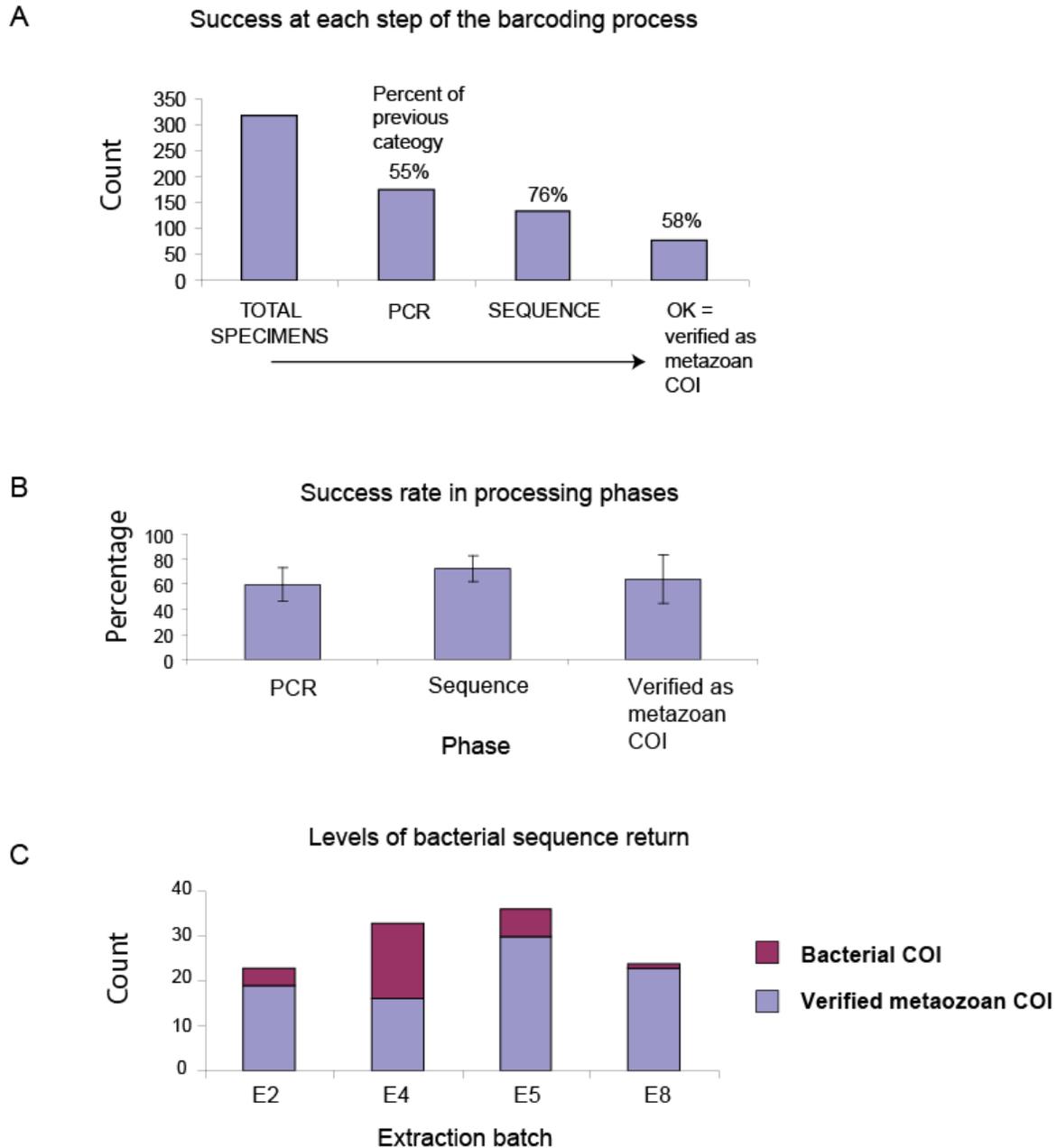


Figure 4. Success rates at each of the major steps in generation of COI sequences for planktonic organisms. Data are from extractions from four plankton collections (E2:16-Nov-06, E4:21-Dec-06, E5:04-Jan-07, and E8:07-Mar-07). (A) PCR success is the generation of a discrete product of correct size, observed on an agarose gel. Sequence success is return of high quality sequence trace files. Verified metazoan sequences are inferred to derive from the target plankter (excluding bacterial sequences). (B) Percentage of successful return at each stage of the barcoding process. Error bars are \pm SD. (C) Proportions of bacterial sequence and metazoan sequences from four plankton samples (extraction plates).

Table 1: Comparison of PCR success rate for focal zooplankton groups from six DNA extraction plates.

<i>total</i>	<i>PHYLUM</i>	<i>PCR SUCCESS</i>	<i>PCR FAILURE</i>
245	Annelida	130	115
87	Crustacea	38	49
131	Mollusca	66	65
<i>SUM</i>		234	229

<p><i>Chi-square test for non-random failure:</i></p> <p>chi² = 2.26</p> <p>df = 2</p> <p>p = 0.323</p>
--

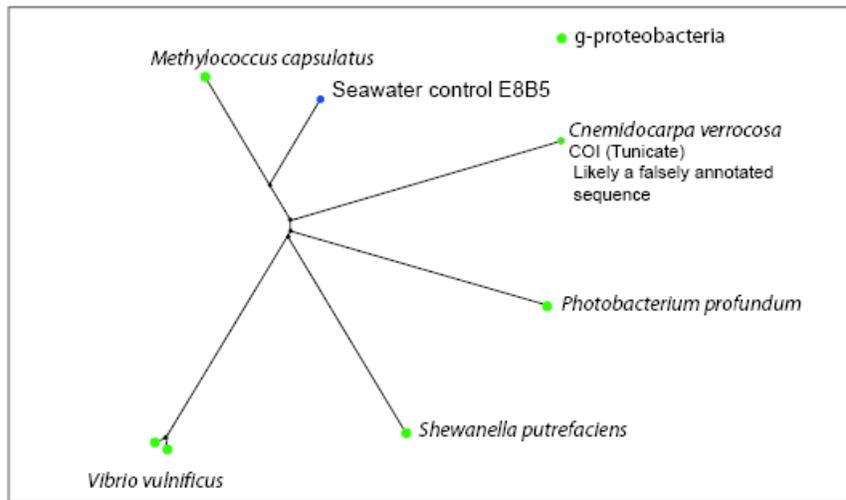


Figure 5. Affinity of a common bacterial sequence obtained from seawater control amplifications. Both adhering seawater and commensal or symbiotic bacteria are likely sources of genomic DNA competing with metazoan DNA in PCR reactions.

2.2.2 Potential sources of bacterial DNA contamination

We hypothesize that microbes in adhering seawater or commensal to the plankter are the primary sources for contaminating bacteria as PCR from seawater controls lead to COI sequences that are similar to environmental marine water bacterial sequences from deposited in Genbank (Fig. 5). Bacterial amplification did not appear to affect extraction batches equally; the extraction conducted on the 21-Dec-06, had a disproportionately high level of bacterial hits. We examined the possibility that holding plankton samples overnight may result to bacterial overgrowth that can lead to a high return of bacterial DNA sequences. The plankton tow from

March 7, 2007 was sorted after 1 h and after 24 h at 12°C. In this particular sample, there was a low rate of bacterial sequence return: twelve sequences were obtained from the 1 h group, and all were metazoan COI; thirteen sequences were obtained after leaving the sample for 24 h, and two (15 percent) were bacterial. The evidence does not suggest that overnight holding of samples greatly affected the unwanted recovery of bacterial sequences. Variation in proportion of bacterial sequences may reflect the temporal dynamics of bacterial populations in Elkhorn Slough.

2.2.3 Construction of a Larval Barcode Database

A Web accessible database run with PHP and MySQL was created by John Witkowski with graphic design by Frances Lynn McMasters (both staff at MLML). The database is a simple tool for keeping track of samples, images, the various steps in the laboratory processing of samples, and the final sequence data. The database is online at <http://invert.mlml.calstate.edu/Plankton>. The database administrator can provide users with password access to enter data online. The Plankton Database includes a specimen/photo ID field, links to multiple images, Notes on Collection and Specimen Handling, and genes sequenced and sequence related notes such as operator's assessment of the similarity to sequences in the GenBank Database (or BLAST match). Screen shots showing database access and organization are shown below (Figures 6 and 7).

<input type="button" value="logout"/>	
Admin	
Input New Sample Data	
Modify Sample Data	
Home	
Plankton DB	
Invert Lab	
MLML	

Sample	
Sample	# 334
ID	6745 Crab Zoea
Photos	6745
Date	1 May 2007
Station	Elkhorn Slough Station 9
Collector Initials	JM
Preserv	Live sample
Ext Plate	EXT5
Position	2F
Notes	
Gene	
Seq Plate	COI
Position	2F
Notes	
Fasta	<pre>>6745_Zooea_E5_2F_07012 9_COI TACTTATATTTTATTTTCGGGAG</pre>
Notes	
Seq Sim	Date: 05/01/2008 (nucleotide BLAST)
Notes	Arthropoda crustacea
GenBank	Unknown species
Notes	Submit to GenBank
<input type="button" value="Submit Info"/>	

Figure 7. The data input page. This entry details sample collection, and the DNA extraction and sequencing plates (sample tracking), and notes on the closest match to a (COI) sequence found in GenBank.

2.3 Discussion

The metazoan COI sequences obtained represent a fairly diverse and novel set (Fig. 8). On average, the sequences show 84 percent similarity to the nearest sequence in GenBank (Fig. 8). This variation (a 16 percent difference among taxa pair) is typical of the level found among different recognized genera of marine invertebrates within taxonomic families; by contrast we found very few plankters were within a 2 percent level of similarity that can often be assigned to a species (Kartavtsev and Lee 2006). Six larvae had sequences with greater than 89 percent similarity to sequences in GenBank (Table 2). These six belong to genera that are known from California: *Obelia* (Hydrozoa), *Membranipora* (Bryozoa), *Cancer* (Crustacea), *Dirona* (Gastropoda), and *Lacuna* (two sequences; Gastropoda), and these generic assignments are very probable. One larva matched *Membranipora membranacea* at 97 percent, and is concluded to belong to that species, consistent with the unique cyphonautes larval morphology.

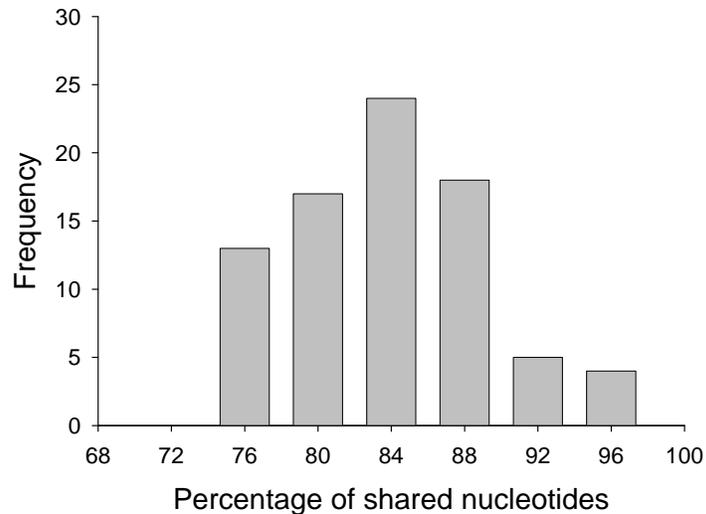


Figure 8. Histogram of genetic distances (percentage similarity) of 81 COI sequences from various Elkhorn Slough Zooplankton groups and the most closely matched COI sequence found presently in the global GenBank nucleotide database.

This pilot meroplankton sequencing project demonstrated that the general concept of DNA barcoding can be applied to invertebrate larvae. The general procedures, from sampling to DNA extraction, sequencing, and data management, can be fit to the small size and diversity of planktonic organisms. A key difference from conventional barcoding projects is that the original sample cannot be physically vouchered, as the entire organism is consumed in the DNA extraction. (However, some larval exoskeletons survive treatment in GeneReleaser and other lysis buffers, and are potentially retrievable). Our concept was to voucher specimens photographically. In principle, photographic vouchers will be more useful to the widest number of uses than physical vouchers because they are viewable on the Web and therefore have the greatest accessibility. Physical vouchers, on the other hand, are most valuable to systematists who need to confirm species identification.

Table 2. Matches of sequenced larvae from Elkhorn Slough with known COI sequences in Genbank. The top match in each case is a probable generic assignments.

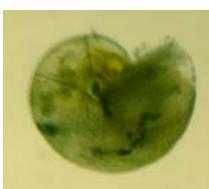
		Top BLAST Match (1-Jun-08)		
		GenBank #	Species	% similarity
Bryozoan larva				
	Sample #	AF147958	<i>Membranipora membranacea</i>	97%
	E56A	AF147963	<i>M. membranacea</i>	97%
	Length	AF147977	<i>M. membranacea</i>	97%
	572 bp	AF147957	<i>M. membranacea</i>	96%
		AF147960	<i>M. membranacea</i>	96%
Hydrozoan (cnidarian) medusae				
	Sample #	AY789913	<i>Obelia dichotoma</i>	95%
	E9C7	AY789914	<i>Laomedea calceolifera</i>	91%
	Length	AY530428	<i>Obelia geniculata</i>	93%
	521 bp	AY530390	<i>Obelia geniculata</i>	93%
		AY530381	<i>Obelia geniculata</i>	93%
Gastropod (nudibranch) larva				
	Sample #	DQ026831	<i>Dirona picta</i>	94%
	E51C	ABI180830	<i>Flabellina verrucosa</i>	82%
	Length	AY345041	<i>Dendronotus frondosus</i>	81%
	609 bp	AY345035	<i>Tethys fimbria</i>	82%
		EF535119	<i>Chromodoris ambiguus</i>	81%
Brachyuran larva				
	Sample #	AF060773	<i>Cancer antennarius</i>	90%
	E62H	AF060769	<i>Cancer gracilis</i>	89%
	Length	AF060768	<i>Cancer novaezealandica</i>	86%
	398 bp	AF060771	<i>Cancer pagurus</i>	85%
		AB211304	<i>Telmessus cheirogonus</i>	84%

Gastropod larva



Sample #	AJ488604	<i>Lacuna pallidula</i>	90%
E56F	AY534458	<i>Zonaria picta</i>	87%
Length	AF129329	<i>Ascorhis tasmanica</i>	86%
498 bp	DQ207181	<i>Zonaria picta</i>	87%
	AF129326	<i>Onobops jacksoni</i>	85%

Gastropod larva



Sample #	AJ488604	<i>Lacuna pallidula</i>	89%
E52H	AF550509	<i>Polinices didyma</i>	86%
Length	AY330824	<i>Adamietta testudinaria</i>	85%
599 bp	AY242949	<i>Adamietta testudinaria</i>	85%
	AY445470	<i>Nucella lamellosa</i>	86%

The absence of a physical voucher and, more importantly, the absence of species descriptions for larvae mean that a larval barcoding project is ultimately dependent on sequences from identified specimens of adults. Although contemplated, we lacked sufficient time and personnel to attempt a survey of adult invertebrates in Elkhorn Slough. *The lack of close matches of retrieved larval COI sequence to entries in Genbank indicates that the California estuarine biota is severely undersampled for COI DNA sequences, the standard gene for DNA barcoding of animals.* Several barcoding projects are underway (see www.barcoding.si.edu) with the goal of barcoding every species on Earth. When complete, obviously all California marine and estuarine invertebrates will be included, and the link between adult and larval sequences will be made. Despite significant investments by private and public (principally Canadian) funding agencies (see www.barcodinglife.org; www.biocode.berkeley.edu), a complete barcode database is not foreseeable in the near future. Subsidiary databases that can later be collected into a global barcode inventory will serve to identification tools for local projects. *A key conclusion of this project is that comprehensive barcoding of adult specimens of Californian marine and estuarine organisms be given high priority by regulatory agencies tasked to assess environmental impacts such as once-through cooling.*

One application of a larval barcode database is to facilitate morphological identification of meroplankton. Once a larval DNA sequence is associated reliably with an adult specimen, the photographic record in the web-based database can be matched to newly collected specimens.

High quality photographs are necessary to distinguish different larvae and to reveal, from multiple entries in the database, the range of possible morphological variation (within and between larval stages). We had difficulty obtaining photographs that have sufficient resolution for this purpose. We used a consumer-level digital camera, but tests with a research grade digital camera (Leica Firewire digital camera) were only somewhat better (producing better color balance). The largest photographic challenge was the blurring caused by rapidly moving live plankters. Locomotion through the field of view and movement of appendages were both problems. Cooling specimens was helpful although possible effect on DNA extraction should be explored. The best technical solution is to use a fast shutter speed while also gathering sufficient light: the sensitivity of most digital cameras is not adequate to accomplish this under normal built-in illumination of typical compound microscopes. Our impression is that high intensity strobe lighting may prove valuable. Another problem is short depth of field inherent in light microscopy optics, which might be solved by digital assembly of focal slices (though this would require a totally immobilized specimen).

We encountered a relatively high frequency of DNA sequences of bacterial origin. The cause of this is universal PCR primers, such as the HCO2198 and LCO1490 primers that we used. Universal mitochondrial COI primers, by definition, target highly conserved regions of mitochondrial DNA in an attempt to give them applicability to taxa that are separated by as much as a billion years of evolution. Because mtDNA is bacterial in its evolutionary origin, these conserved regions are found in bacterial as well as eukaryotic genomes. Bacterial contamination is probably found in almost every biological sample that is not specifically cultured in axenic conditions. Under normal conditions, however, DNA from the target organism far exceeds that of symbiotic, commensal, or truly contaminating bacteria. When multiple homologous DNA templates are present, PCR is a competitive process with the advantage going to the more abundant template. When the intended target DNA is in low abundance (in this case, because the planktonic organisms are very small), unintended templates may be a significant portion of the total DNA. We hypothesize that Elkhorn Slough waters were particularly rich with bacteria whose COI genes happened to be a good fit to the PCR primers we used.

We considered that our protocol of holding plankton overnight may have contributed to the bacterial contamination problem, but one limited test in which we compared samples sorted on the day of collection and on the following day did not show a striking difference in bacterial DNA sequences. However, in this test both samples had a low rate of bacterial sequences. We do not, therefore, consider this test as definitively ruling out culturing as a source of bacterial contamination. Immediate on-boat preservation of samples in ethanol appears to be a good option for limiting the opportunity for bacterial growth. However, this will interfere with photographic documentation because specimens will be contracted and lose color. Also, fragile and gelatinous plankton will not hold up well in ethanol.

In cases where bacterial sequences make a large proportion of recovered DNA sequences, novel approaches to limit those extraneous sequences are needed. We recommend that future research address this problem. One approach might be to develop more specific primer sets; i.e., annelid, crustacean, or molluscan sets. Since larvae were manually sorted, they easily could be

aggregated by higher taxon and different primers used in PCR. However, it is a significant challenge to design phylum or class inclusive primers for a gene that is sufficiently variable to be useful for identification at the species level. It is for this reason that the LCO/HCO primer set is widely used- there are few good alternatives that target COI, the gene that is most commonly used for DNA barcoding. As a solution to this dilemma, templates might be enriched for eukaryotic DNA by targeting conserved regions that flank the entire COI gene, or indeed by amplification of the entire mitochondrial genome. Once enriched, the standard LCO/HCO primer set may target metazoan sequences more efficiently.

Overall, we experienced a ~20 percent success rate in obtaining COI DNA barcodes from larvae. Beyond the losses due to recovery of bacterial sequence, we experienced ~50 percent PCR failure and ~50 percent sequencing failure. While PCR is notoriously fickle, we not have a good explanation for the low rate of sequencing success except perhaps inefficiency of the sequencing facility that had some instrument failures during our project period. In general, we developed low-cost yet medium-throughput methods for extraction, PCR, PCR cleanup, and sequencing, with apparent trades between cost and efficiency. Thus, from the perspective of testing the utility of DNA barcoding, we are not discouraged by the low rate of success we experienced because the reliable high-throughput methods exist and their costs continue to decline. Economy of scale in larger scale barcoding projects will make these methods cost effective.

2.3.1 Future prospects and recommendations

The concept of DNA barcoding plankton is valid. The key obstacles are workflow bottlenecks at the plankton-sorting step and a need for better photographic methods. The last is solvable with current technology and investment in appropriate instrumentation. However, the laborious sorting of plankton individually is always going to be a critical limiting step, especially when a photographic record of each plankter is needed. We can envision a time when the adult sequence database is rich enough that sequences drawn from bulk plankton analyses will be sufficient to characterize a plankton sample. Real-world applications of bulk zooplankton analyses are still forthcoming, but traditional approaches from microbial ecology or approaches using newer technologies can be contemplated. Traditionally, bulk bacterioplankton are extracted, amplified, cloned and sequenced, and this method could be applied to zooplankton. Yet, biases in each step make this problematic for full biodiversity assessment. An alternative is exhaustive sequencing to capture maximum diversity in a plankton sample. The advent of massively parallel sequencing MPS technology (pyrosequencing, for example) makes the sequencing of millions of templates from a PCR product possible. As the cost of MPS decreases, this seems to us the most practical approach to analysis of entire plankton samples.

The usefulness of hand-sorted, sequencing of clone libraries, or of exhaustive MPS sequencing are all dependent on the existence of a substantially complete estuarine and marine life barcode database. Because we predict that the development of low cost MPS technology is imminent, immediate effort is best directed at development of an adult COI barcode database for organisms likely impacted by once-through cooling.

CHAPTER 3:

Quantitative PCR in plankton samples: environmental variables and development of brine shrimp *Artemia franciscana* as organismal standard.

3.1 Introduction

Identification and counting of zooplankton is one of the most laborious and costly elements of plankton research. As such, it is often a limiting step in the scope of research that can be feasibly attempted. Additionally, early life history stages of many holoplanktonic and meroplanktonic species cannot be identified by morphology because the adults to which they correspond are not known. As in the medical field, polymerase chain reaction (PCR) based methods are likely to assume and extend the role played by microscope based methods in plankton studies because of the general versatility, specificity, increased throughput, lower labor costs, and higher detection power (Ginzinger 2002, Giglio et al. 2003). Quantitative PCR methods (referred to as QPCR) utilize the rate of increase of a specific PCR product to infer starting target DNA copy number or mass in the reaction. Central to the increasing popularity of QPCR is the use 'real time' DNA-reporting fluorescent dyes (including ethidium bromide, SYBR Green I) and fluorescence emitting reporter probes, coupled with hardware and software to monitor amplification. DNA and RNA, when coupled to a reverse transcription step, can be used as targets for QPCR. RNA and genomic DNA templates have been successfully used to detect toxic algal organisms in water samples and sediment (Saito et al. 2002, Popels et al. 2003, Coyne and Craig Cary 2005, Coyne et al. 2005, Bowers et al. 2006), suggesting PCR is sufficiently specific and robust to be used as a general procedure in marine environments.

Quantitative PCR has exceptional potential for the study of natural plankton communities, expanding the effective volume of the environment which can be comprehensively analyzed in comparison to strictly microscope-based effort. However, there are a number of systematic and operational issues in reliably applying PCR to quantify target species in environmental samples, including DNA extraction efficiency, and the sensitivity with which (dilutions of) extracted DNA can be probed for the presence and quantity of a target organisms in the PCR reaction. Community samples contain genetic diversity that may potentially result in spurious amplification, and Type I (false positive) detection error. Detectable PCR amplification and rate of template doubling (sources of type II error) are both affected by the presence of co-extracted PCR inhibiting substances, including non-target genomic DNA (which may interact with primers), and chemical inhibitors (Wilson 1997, Vadopalus 2006, Wintzingerode et al. 1997), in addition to typical sources of variation in PCR experiments (e.g., suboptimal Mg^{2+} concentration, pH or annealing temperatures). Humic acids, large diverse organic compounds (Zipper et al. 2003) present in aquatic sediments strongly affect PCR reaction success (Wintzingerode et al. 1997). Inhibition may occur because of genomic DNA and a diverse array of coextracted chemicals (including pigments and secondary metabolites) which have not been analyzed systematic for effects on PCR.

We used the brine shrimp species *Artemia franciscana* (Crustacea, Anostraca) to explore the potential performance of QPCR when applied to a zooplankton species in marine water samples. Free-swimming brine shrimp are readily hatched (as 0.4-0.5 mm nauplii in *A. franciscana*) from cysts which encase individual embryos in arrested developmental state (Clegg 1962, 1974). All *Artemia* species occur in settings with above normal seawater salinity, such as salt lakes and evaporation pans (Browne and MacDonald 1982, Perez et al. 1994, Sarabia et al. 2002, Baxevanis et al. 2006) and do not occur in freshwater and marine communities. As nauplii can easily be added to plankton samples prior to the DNA extraction, brine shrimp may provide an excellent positive control for providing confidence in detecting and quantifying species of interest in plankton-based QPCR surveys.

We developed *A. franciscana*-specific primers, for two high copy number genes, the nuclear *ribosomal 18S RNA* gene and the mitochondrial *cytochrome c oxidase subunit 1* (COI) gene. We then tested the reliability and quantitative ability in real-time detection by two QPCR systems. The first uses SYBR Green I dye, which fluoresces upon binding double-stranded DNA (dsDNA), to report total (non-specific) increase in dsDNA over the course of PCR. The second system was the TaqMan assay, which utilizes 5'-3' exonuclease activity of *Taq* DNA polymerase to digest a dual-labeled oligonucleotide probe that anneals to PCR products, thereby releasing a fluorescent molecule from a quenched state (Holland et al. 1991, Livak et al. 1995, Heid et al. 1996). The 'threshold cycle' (Ct, or 'crossing cycle') principle is the most widely used basis for quantification in real time QPCR. Ct is defined as the PCR cycle in which amplification-related fluorescence signal becomes greater than a designated level of starting or 'background fluorescence' signal. Operationally, Ct is viewed as a horizontal line on fluorescence by cycle plots above which fluorescent signal exceeds a minimum background level, and can be thought of as the cycle in which the reaction begins exponential increase in fluorescence. Ct values have a linear relationship to \log_{10} of DNA quantity when applied at any point within the exponential phase of the PCR reaction (Larionov et al. 2005). Standard curves are generated for quantification using a DNA dilution series. Quantities can be expressed as gene copy number per reaction (using a purified DNA source such as a plasmid marker, or a total mass of genomic DNA containing a certain number of gene copies, ranging from two per single genome mass for a single copy number, diploid gene to 10s or 1000s of copies per genome in the case of multicopy mitochondrial and ribosomal genes).

We undertook experiments to assess the specificity of the *A. franciscana* primers in samples of marine plankton DNA, and the degree of inhibition posed by a set of factors, including increasing concentration of genomic DNA from marine plankton samples, and herring sperm DNA (as a pure DNA reference source), and Ct scaling difference due to reporting system. The accuracy of recovering the DNA content for counts of *A. franciscana* nauplii, or mature brine shrimp, was examined by addition of brine shrimp to the sample prior to DNA extraction.

3.2 Materials and Procedures

3.2.1 Brine shrimp stock

The major North American brine shrimp species, *Artemia franciscana*, which has diploid, sexually reproducing populations, was used for experiments. Cysts were supplied by M&M

Suppliers, Bothwell, WA, USA. Cysts were added to filtered aerated seawater at 24°C. Nauplii were collected by pipette after 24 h. Adults were obtained by culturing for 35 days with *Spirulina* algae powder (M&M) provided as food.

The reference sequences used for primer design are the *A. franciscana* 18S ribosomal RNA sequence reported by Weekers et al. (2002; Genbank acquisition: AJ238061) and the *A. franciscana* mitochondrial genome reported by Perez et al. (1994; X69067). COI and 18S nucleotide sequences of experimental brine shrimp populations were examined by sequencing using PCR primers LCO1490 and HCO2198 for COI (Folmer et al. 1994), and 18e and 18L for 18S rRNA (Struck 2005), and showed a close (>99 percent) similarity to the reference sequences.

3.2.2 Probe design

PCR primer sets were designed to amplify short (c. 100 bp) fragments of the COI and 18S rRNA genes. Primers were selected using Primer 3 software (Rozen and Skaletsky 2000, Koressaar and Remm 2007). Expected melting temperatures were calculated using the formula of (Owczarzy et al. 2004) assuming a monovalent (KCl) cation reaction concentration of 50 mM. PCR primers were designed with a target melting temperature of 60°C. For TaqMan assay, an internal hybridization probe was designed with an expected melting temperature of 70°C to expose it to 5'-3' degradation during product extension. The hybridization probe-template duplex is expected to have a slightly higher melting temperature than predicted, because 6-FAM has a stabilizing on DNA duplexes (Kutyavin et al. 2000). Primers and TaqMan hybridization probes were synthesized by Integrated DNA Technologies. 6-carboxyfluorescein (6-FAM) and a FRET electron transfer quenching molecule (Blackhole quencher™) were attached to 5' and 3' termini of hybridization probes, respectively. 6-FAM was chosen because it has similar excitation and emission wavelength maxima to SYBR Green I (Bengtsson et al. 2003, Zipper et al. 2004).

The specificity of candidate primers was experimentally determined by endpoint PCR using the primer pairs to amplify DNA extracted from plankton samples collected in Elkhorn Slough (described below). PCR reactions consisted of 1.5 mM MgCl₂, 1X reaction buffer (Invitrogen), 125 μM dNTPs and 0.5 units Taq Polymerase in an 18 μl reaction volume. Plankton DNA was exponentially diluted and added to the reaction at four concentrations (432 – 0.432 ng/reaction). Reactions were run for 30 cycles using a range of annealing temperatures: extension (72°C), annealing (50, 55, or 60°C), and melting steps (94°C) of 30s each. *Artemia* DNA was added to positive control reactions to test for PCR functionality, while negative controls (included in all experiments) contained no template DNA. Reaction products were separated electrophoretically on 3 percent MetaPhor high resolution agarose (Cambrex) and visualized under UV using ethidium bromide.

3.2.3 Quantitative PCR Methods

Real-time QPCR reactions were conducted using the iCycler Real Time thermal cycler (BioRad), monitoring with the FAM/SYBR Green I detection filter. Reactions were conducted using a 20 μl reaction volume in 96-well plates, sealed with optically clear tape (Thermo Fisher Scientific). Data were collected using a standard three-step PCR amplification for SYBR reaction and a two-step amplification for TaqMan amplifications (Fig. 1). Absolute™ SYBR Green I pre-Mix

containing SYBR Green I and Fluorescein for normalization was used for SYBR reactions; and Absolute™ QPCR Mix for TaqMan reactions. The reagents included thermally activated Taq polymerase and were supplied as 2X reaction mixes by Fisher-Thermoscientific. Markers were assayed separately, simplifying baseline setting. The threshold cycle, or Ct, was defined automatically using the Base Line Subtracted Curve Fit method. *Artemia franciscana*-negative reactions were analyzed to quantify the potential for false positive signaling in each system.

All TaqMan reactions showing threshold crossing (detectable amplification) were included in analyses. Ct values of negative control reactions, to which no *A. franciscana* DNA was deliberately added, were compared to evaluate minimum reliable detection quantities of target. From this comparison, we defined a failed reaction as one that showed no threshold crossing after 50 cycles of temperature cycling. The SYBR Green ('SG') reaction allows detection of secondary products, such as primer-dimer molecules. Primer-dimer molecules and any spurious PCR products have a different (lower, in the case of primer-dimer) melting temperature (T_m) than target PCR products (Giglio et al. 2003) that is readily detected in by a melting curve assay applied after PCR. Primer-dimer artifacts were found in some reactions with low (less than 10^{-5} ng/reaction) concentrations of target DNA and in some no-target controls, representing less than 10 percent of trials.

3.2.4 Detection and quantification of *A. franciscana* DNA in Plankton Sample DNA

The dynamic range and accuracy of detection by different gene-reporter systems was studied in the presence of DNA extracted from marine plankton collections. DNA extracted from a plankton sample is referred to as 'PDNA'. Plankton sampled between April and August, 2007 at Elkhorn Slough, California, a tidal marine-dominated estuary and nearby marine waters of Monterey Bay, California. Tows were semi-quantitative using an 83 μ m mesh net by boat (net diameter 0.5 m; tow duration 5 min; speed: ~ 1 knot, or 0.514 ms^{-1}). Live plankton was concentrated on 10 μ m nytex filter, rinsing with filtered, autoclaved seawater, and then stored at -80°C . Extractions were conducted prior to October 2007. A fraction of the material obtained in any one tow was used for DNA extraction.

3.2.5 Whole plankton DNA extraction 1

Solid material was frozen using liquid N_2 and ground by mortar and pestle, and then digested using pro-K (20 mg/ml). DNA was extracted on gravity-driven silica affinity columns (Qiagen Genome-Tip 500/G) according to the manufacturer's specifications and eluted into 200 μ L nuclease-free ddH₂O. Effects of increased PDNA concentration were examined using two plankton samples collected in Elkhorn Slough, with DNA content elevated to 400 ng/reaction. In another treatment, herring sperm DNA (Promega) was added at 800 ng/reaction to examine potential inhibitory effects of a very high background of DNA from a dominant organism.

3.2.6 Enumeration of *A. franciscana* nauplii in a plankton sample

Different numbers of nauplii were added to marine plankton samples and prepared for QPCR using a second plankton sample (Fig. 1). The plankton for this experiment was collected from Elkhorn Slough (MLML Stn 9, August 2007) and stored at -80°C for 24 h. The sample was then thawed and divided into ~ 20 mg wet weight mass aliquots for DNA extraction by Qiagen DNeasy kit Qiagen DNA easy columns. *Artemia franciscana* were obtained at 20 h after cyst

immersion. Some plankton samples were spiked with one mature male brine shrimp (bearing claspers) to approximate the order of magnitude difference in total DNA content occurring in free swimming stage of the brine shrimp life cycle.

Table 3: *Artemia franciscana*-specific PCR primer and TaqMan hybridization oligonucleotide sequences

Gene	Primer/probe name ^A	Sequence	length (bp)	Estimated T _m ^B	Product length (bp)
18S	Af18S-1298f	5'-GTT GGT GGA GCG ATT TGT CT-3'	20	65°C	129
	Af18S-1387r	5'-AGA GCG ATC CAC CAC TAG GA-3'	20	66°C	
	Af18S-1326TMf	5'-6-FAM-CCG ATA ACG AAC GAG ACT CTA GCC TGC- Quencher-3'	27	75°C	
COI	AfCOI-1911f	5'-TCG CTT CAT TTA GCT GGA GTT TC-3'	23	67°C	104
	AfCOI-2043r	5'-GGT GAT TCC TAC TGC TCA GAC G-3'	22	67°C	
	AfCOI-2006TMr	5'-6-FAM-CCG TCA ATA GAT ATT GAC TGG GGT CGT- Quencher -3'	27	72°C	

^ANumbering indicates position of 5' nucleotide in alignment with the following reference sequence.

18S (*Artemia franciscana*): Genbank acquisition number AJ238061 (Weekers et al. 2002).

COI (*Drosophila yakuba*): Genbank acquisition no: NC_001322 (Clary and Wolstenholme 1985).

^BOligonucleotide melting temperature (T_m) was calculated using salt correction formula of (Owczarzy et al. 2004).

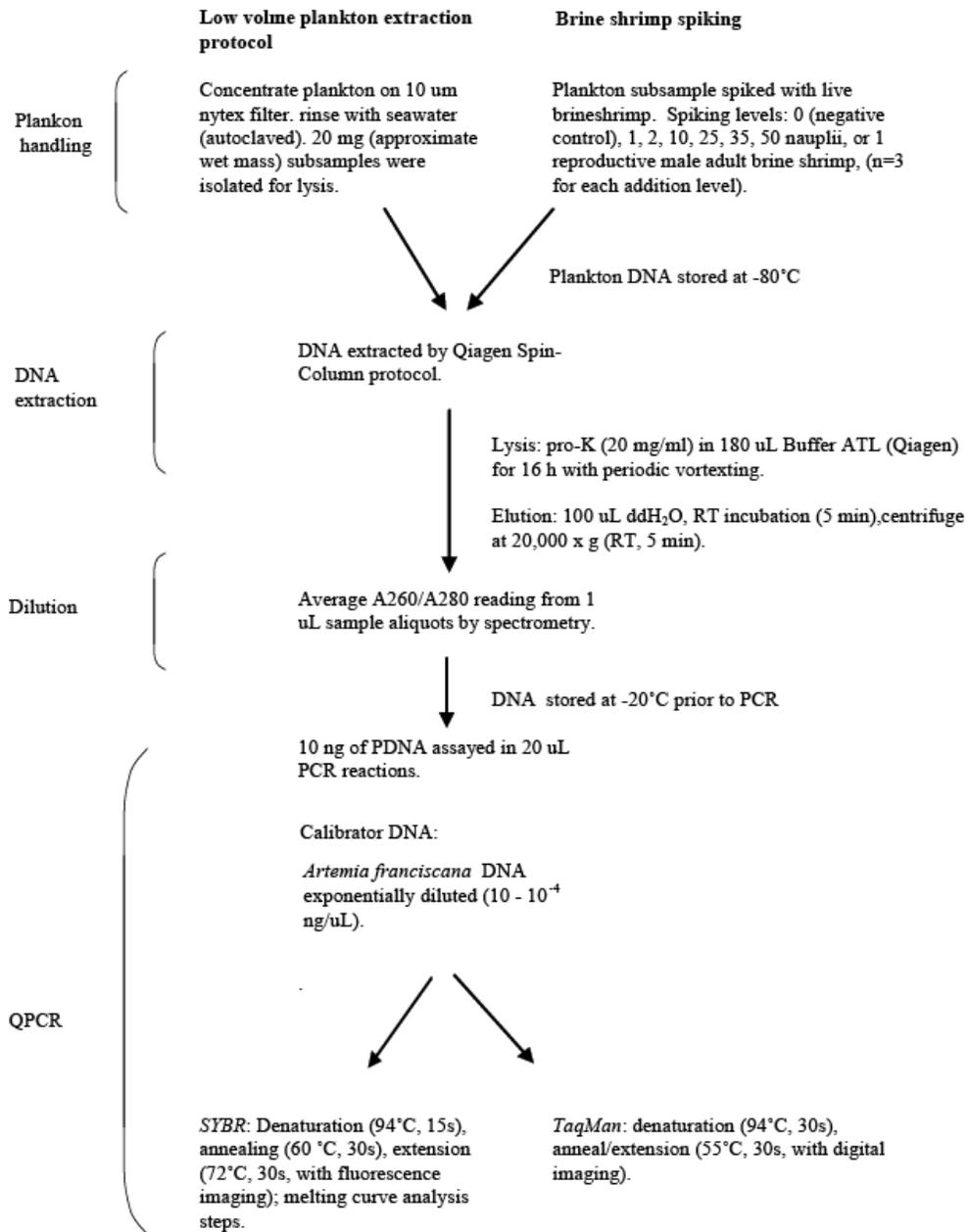


Figure 9. The plankton DNA extraction and QPCR protocol used in the nauplius spiking experiment.

CHAPTER 4: Results

4.1 Overview

Our first goal was to develop a sensitive and specific assay for specifically amplifying and measuring *A. franciscana* DNA in a background of plankton DNA. Primer specificity was achieved by eliminating candidate primers that yielded PCR products (seen on agarose gels) when used in PCR with plankton DNA presumed to lack *Artemia*. Analysis of a total of five candidate 18S primer pairs yielded one *Artemia franciscana* specific pair. Analysis of five COI primer pairs yielded two specific pairs. All QPCR experiments were conducted in the SYBR Green I format (measuring total DNA synthesis) and TaqMan format (Table 1) using one primer pair each for amplification of 18S and COI. The QPCR treatments are referred to as: SG-18S, SG-COI, TM-18S and TM-COI.

PCR reaction efficiency (E percent) an estimate of per-cycle percentage of doubling of the template was determined using the formula: $E \text{ percent} = (10^{(-1/\text{slope})} - 1) \times 100 \text{ percent}$ using the slope from standard curve plots (Ct versus log genomic DNA mass). Taqman reactions showed lower PCR efficiency compared to SYBR green: $E_{SG-18S} = 99.7 \text{ percent}$; $E_{SG-COI} = 102.0 \text{ percent}$ $E_{TM-18S} = 89.4 \text{ percent}$ $E_{TM-COI} = 87.9 \text{ percent}$. Consequently, Ct (the amplification detection threshold) was reached earlier in the PCR process using SYBR Green (Fig. 2).

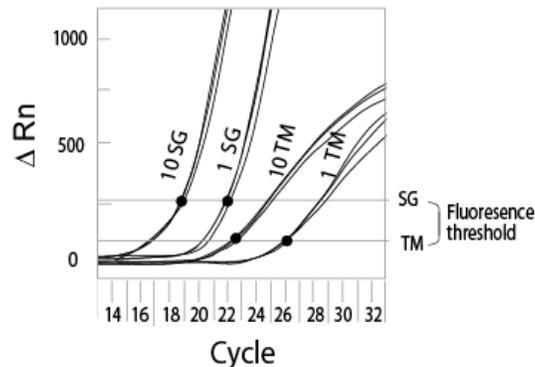


Figure 10. QPCR traces for amplification of 18S rRNA from 10 ng and 1 ng of *Artemia franciscana* genomic DNA (three replicates) using SYBR Green 1 (1SG and 10SG) or Taqman (1TM and 10TM) probes from separate reactions on a BioRad iQcycler. ΔRn is the (fold) difference in signal relative to automatically established threshold fluorescence.

We next examined the effect of different plankton DNA (PDNA) on Ct and reaction success. Sample 1 was from Elkhorn Slough and consisted of abundant zooplankton with little obvious algal material or sediment. Sample 2 was collected within the shallow upper portion of Elkhorn Slough and contained organically rich sediment and detritus attached to the bottom due scraping of the bottom surface by the net (Mackie pers obs). Sample 3 was collected 1 mile offshore in oceanic Monterey Bay. This sample contained abundant microalgae algae, giving it a

green color. Although the samples used for DNA extraction were obviously different in community composition, the final A_{260}/A_{280} ratio for DNA extracted from each sample was greater than 2.0 (unpubl. data), which is indicative of a nucleic acid sample lacking protein. Insufficient DNA was obtained from sample 3 for inclusion in experiments at high DNA concentration.

At five ng of the plankton community DNA per reaction, the lowest PDNA background concentration used in experimental trials, there was little difference in Ct values with PDNA values and with *A. franciscana* DNA diluted simply in water (Fig. 3). *Artemia franciscana* was distinguished over the range of 10^{-5} to 10^0 ng per reaction. Increased variance in Ct was apparent at 10^{-4} and 10^{-5} ng levels of target genomic DNA in the case of the TM-COI system. Negative control (-*A. franciscana* DNA) reactions were included, indicating occasional, late cycle threshold crossing events, in each case below the expected Ct value for 10^{-5} ng of the target (Fig. 3).

Although each QPCR system was able to detect *A. franciscana* genomic DNA over the seven-orders of magnitude, reaction success decreased from 100 percent at upper concentrations to 50 percent or less at 10^{-5} ng dilution level, regardless of amplicon or signal format (Fig. 3).

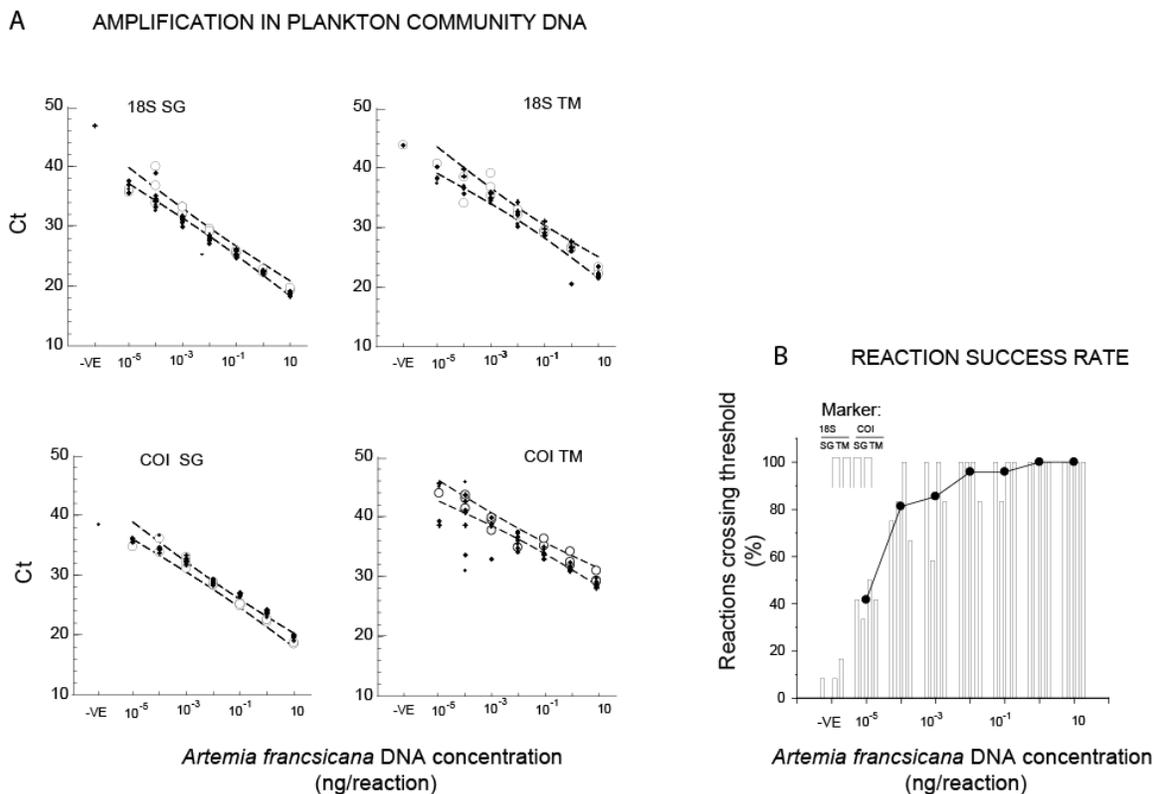


Figure 11. Threshold cycle (Ct) plots for amplification of an *Artemia franciscana* DNA diution series in in the presence of 5 ng reaction⁻¹ of DNA from three plankton community samples: 1 (●), 2 (+) and 3 (◆); or neat dilution in ddH₂O (○) by different QPCR markers (A). Broken lines are the 99% confidence interval estimated by linear regression in water. Plot B shows the total percentage of reactions showing amplification signal (threshold crossing by PCR cycle 50) at different

background DNA concentrations, comparing the frequency of signaling in control PDNA samples that lacked *A. franciscana* DNA (-VE).

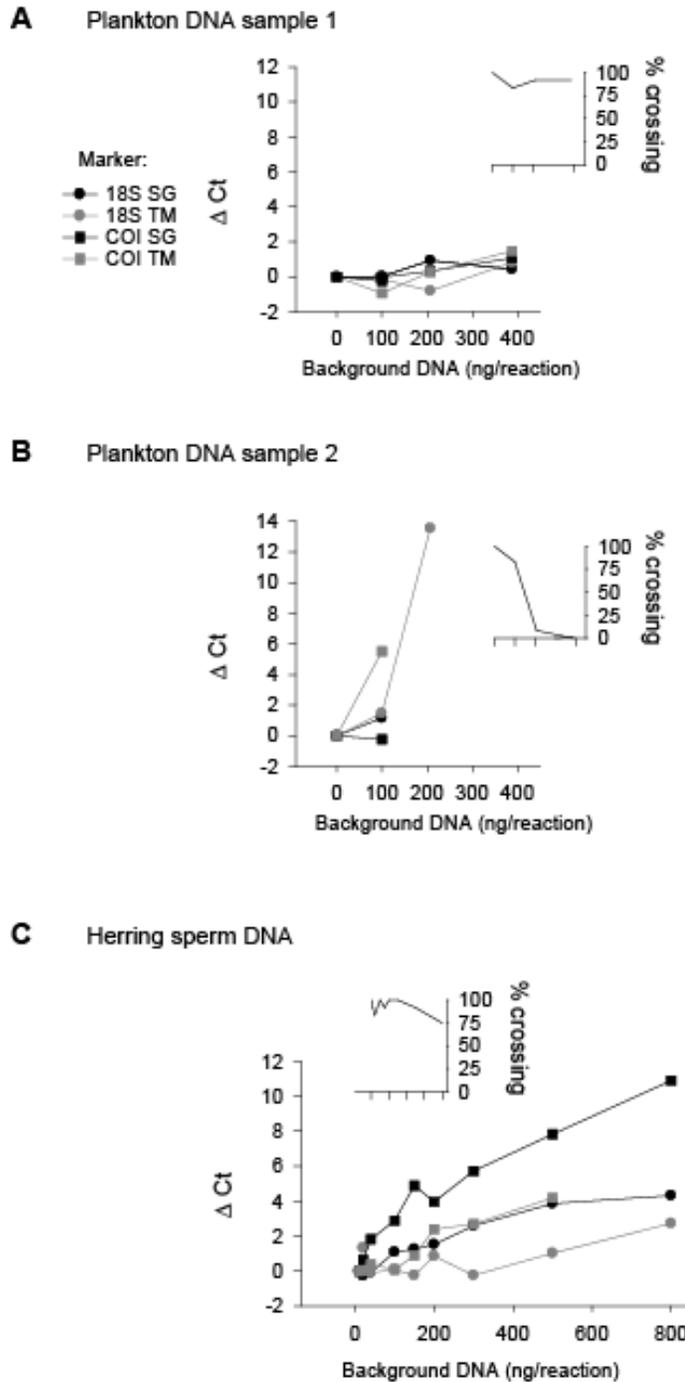


Figure 12. Effect of increased background DNA on amplification of *Artemia franciscana* DNA at 10^3 ng reaction⁻¹. Large plots show deviation in threshold cycle (Δ Ct) from a reactions with no background DNA. Inset graphs show the total percentage of reactions with threshold crossing, indicating amplification success. (A and B) Plankton community DNA samples 1 and 2 were collected at Elkhorn Slough, California. There was little inhibition due to PDNA 1, whereas complete inhibition by PDNA 2 was observed above a threshold concentration. (C) Herring sperm DNA caused PCR inhibition variously for the each marker-reporter system combination.

To examine QPCR effects of increasing PDNA sample concentration (which may be a desirable strategy for expanding the volume of the environment that can be sampled by QPCR) samples A and B was increased in concentration to 400 ng/reaction. The elevation of PDNA concentration sample generally delayed Ct and increased the rate of reaction failure (Fig. 4). Because reactions contained a constant level of *A. franciscana* DNA (0.01 ng reaction⁻¹), Ct values were expressed as deviations from a starting Ct value corresponding to the neat dilution. Background A inhibited with no effect on reaction success rate (Fig 4A). The averaged slope and standard deviation of Ct regression lines was $4.6^{-3} \pm 2.2^{-3}$, corresponding to an estimated increase of approximately 1 ct unit increment for each 100 ng of background sample DNA concentration. The obvious effect of Increasing background PDNA B was reaction failure at concentrations >100 ng (Fig 4B). Herring sperm DNA, a highly purified DNA background showed substantial Ct delay with little reaction failure (25 percent. at 800 ng/ reaction) (Fig 4C). The degree of inhibition varied widely for different markers (highest: COI SG; lowest: 18S TM). This suggests that the strength of inhibition depends upon idiosyncratic interactions between primers, templates, and reporting systems.

4.1.1 Brine shrimp enumeration

Our major goal was to determine the accuracy of QPCR systems for determining quantities of brine shrimp added to a marine plankton sample prior to extraction. All samples were assayed at 10 ng of PDNA per PCR reaction. (This resulted in Ct definition for one nauplius of 24.1-27.4 for the SYBR Green systems, and 29.2-34.4 for TaqMan systems.)

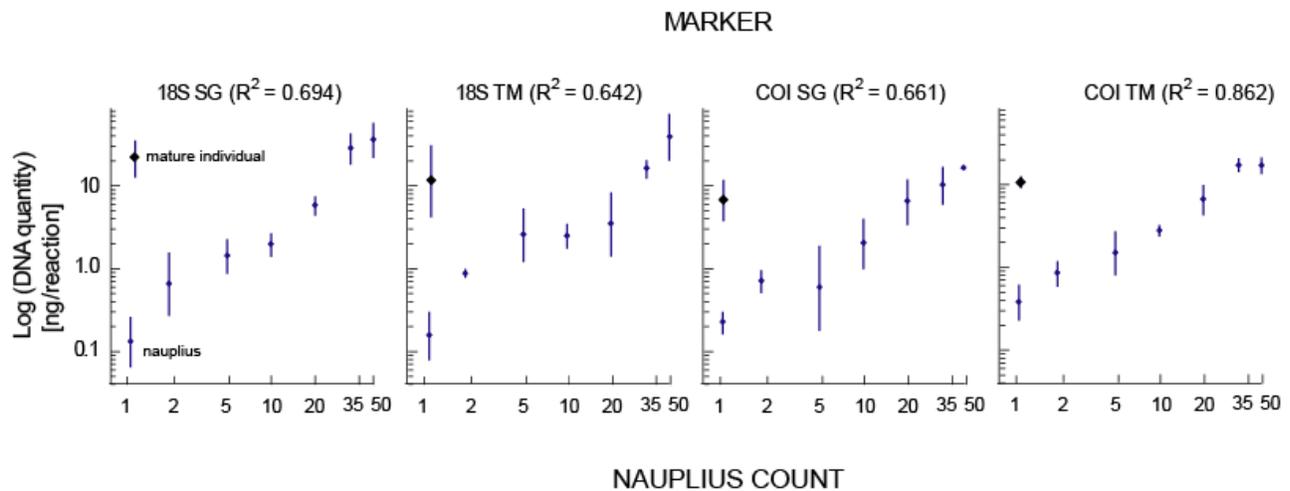


Figure 13. Brine shrimp enumeration within a plankton sample. Estimates of *A. franciscana* DNA in PCR reactions containing 10 ng of plankton community DNA versus number of nauplii which were added to each sample prior to DNA extraction. Alternatively one mature male individual was added to a reaction to show approximately the magnitude of variation in DNA content over the life cycle of this organism. Each sample level was assayed in triplicate. Error bars are ± 1 SE of the mean. Linear regression coefficients (R^2) are shown for nauplius count versus untransformed DNA quantities.

There was a well supported linear relationship with the number of nauplii added to each extraction and estimated *Artemia* DNA: the overall relationship between $\log_{10}Ct$ determined DNA content (y) and nauplius count (x), pooling estimates from four marker analyzed in individual reaction runs was by the order regression was: $y = 0.1589x - 0.0958$ and had a regression coefficient, R^2 , of 0.791. Regression coefficients from individual markers/individual reaction runs ranged from 0.694 to 0.862 (Fig. 5). Graphical presentation of the estimated DNA quantity and nauplius count using log-transformed axis, as in Fig. 6., illustrates that it was possible to distinguish the DNA content of one and two nauplii in plankton samples with the given level of sample replication ($n = 3$), regardless of the reporter or marker used. In relative terms, the quantity of DNA in a mature individual was equivalent to 20-35 newly hatched nauplii (Fig 5.). The linear relationship between nauplius numbers and estimated DNA content provides for estimation of nauplius numbers in assays of unknowns. Clearly, however, 21 percent of variation was unexplained in this regression, therefore 90 percent confidence intervals can be calculated to determine the likely range of nauplii in unknowns.

4.1.2 Estimation of absolute 20h nauplius DNA content

Linearity in estimation indicates that, although the quantity of PDNA returned at the DNA extraction varied by ~4 fold in yield (Fig. 6), *A. franciscana* genomic DNA was apparently homogeneously distributed in 'recovered' and 'lost' extraction portions. To assess the accuracy of plankton-based QPCR in absolute terms, we determined the level of total DNA at the 20 h stage of *Artemia* development within the PDNA spiked series, utilizing all estimates by dividing by input number of nauplii. All samples were multiplied by an independent experimental dilution factor which ranged from 1.93×10^3 - 5.19×10^3 to 10 ng per PCR reaction. The mean estimate of DNA per *A. franciscana* nauplius in our experiment was 6.14 ± 3.14 ng.

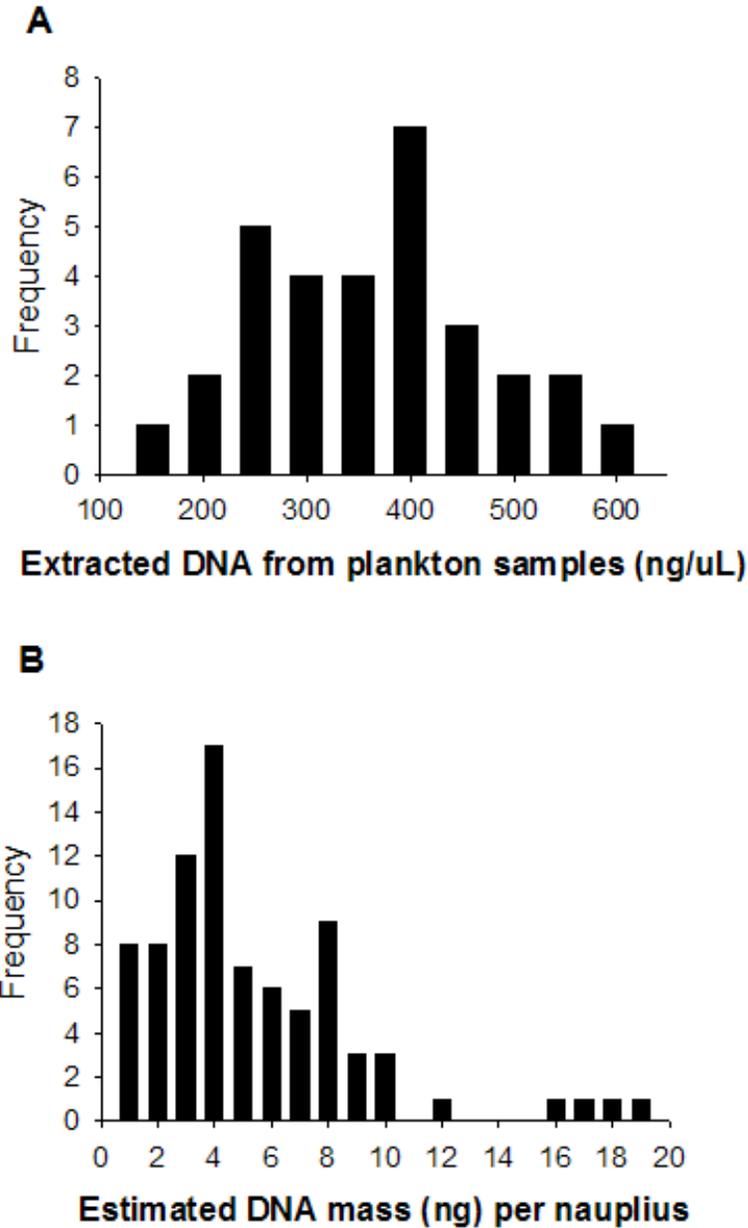


Figure 14. Distribution of total plankton DNA total extracted from Qiagen Minispin columns (A), and the final range of DNA mass estimates per nauplius (B) from the spiking experiment.

We lack information to test explicitly for DNA extraction inefficiency, a possible source of systematic underestimation in QPCR. However, we compared a previous measure of the 20h developmental stage nauplius DNA content for this species of 6.1 ± 0.5 ng per animal made previously by Southern analysis (Fig 3B in Vallejo et al. 1996). The striking correspondence to our estimate suggests no systematic loss due to extraction efficiency, but rather that variation in net DNA extraction totals for brine shrimp genomes was distributed around the true mean according to central limit theorem.

Despite the apparent quantitative outcome, estimates of the DNA total in the nauplius spiking experiment had substantial deviation (Fig. 6B). The overall coefficient of variation, a standardized measure of dispersion, for the nauplius counting experiment was 0.51. The estimates were non-normally distributed, presenting a tail of high-end estimates (Fig 6B). Generalized Linear Model ANOVA was conducted using log-transformed estimates of DNA (Table 2). A significant portion of estimation variance was due to variation among DNA extractions ($P = 0.028$). Reaction run (which was analogous to the marker system used), had a marginal but non-significant effect ($P = 0.056$). The number of nauplii was not a significant source of estimation variation ($P = 0.958$), nor was the actual concentration of eluted DNA obtained per sample. The later factor was tested in a separate, single factor ANCOVA ($P = 0.612$; not shown).

Table 4. Analysis of variance in estimation of single-nauplius DNA content in the plankton spiking experiment

Source	SS	df	MS	F-ratio	P
DNA extraction	16.775	19	0.883	1.935	0.028
Nauplius addition level	0.001	1	0.001	0.003	0.958
Reaction run ¹	3.649	3	1.216	2.665	0.056
Error	27.380	60	0.456		

1 A different marker was used in each run: 18S SY, 18S TM, COI SY, or COI TM.

4.1.3 Distinguishing false positives from reliable signal

For true field implementation it is necessary to examine present data indicating the ability of QPCR to successfully recognize the presence of false positive reaction signals. Threshold crossing in negative reactions may be caused by template contamination, non-specific amplification, signaling artifacts, or primer-dimer production in SYBR Green I format. Threshold crossing occurred in one (COI-TM), one (18S-TM, COI-SG), or two (18S-SG) cases in the first experiment. The negative reaction Ct values ranged from 39-47, which was above the threshold cycle determined for 10^{-5} ng level of target template in each case. In the nauplius counting experiment, threshold-crossing signal, when present in our negative control reactions, was quantified as $< 10^{-4}$ ng of *A. franciscana* genomic DNA equivalents in all cases. Some samples from SG reactions were cloned and sequences examined, indicating that these consisted firstly of sequenced primer-dimer repeats; and secondly, *A. franciscana* DNA, indicating cross transfer of template (data not shown).

4.2 Comments and recommendations

Internal standards are commonly included following DNA extraction for different operational aims: to identify false negative results due to PCR inhibition (Saito et al. 2002, Coyne et al. 2005)

and to maximize DNA extraction efficiency. This qualitative need is particularly great in plankton based studies. Detecting/quantifying individuals in a water body involves a series of dilutions that are strongly dictated in scale by the seston, or density of physical suspended matter, and practical limitations including the volume of matter which can be used for DNA extraction, chemical limitations to the fidelity of PCR for quantification.

Because of the culturing ease, availability of the genetic stock, and reliable exclusion of the species from marine and freshwater habitats we see the use of brineshrimp as useful 'organismal' standard in plankton studies because PCR quantification of the brineshrimp gene target can incorporate experimental variations, including extraction efficiency, genomic target complexity, and effects of co-extracted substances. Primers developed for 18S and COI appear to be similarly reliably and precise for quantifying *A. franciscana* DNA. *Artemia franciscana* cysts are readily available and inexpensive, although, we and other laboratories (Marco et al. 1991) have found that it is important to check batches carefully as some products advertised as *Artemia franciscana* contain cysts of another species, in particular the parthenogenetic species *Artemia salina*.

To be quantitative in an absolute sense, QPCR requires an efficient and reproducible extraction protocol (e.g. (Böstrom et al. 2004), or alternatively the use an external control to absorb effects of variation in extraction efficiency via relative quantification. Relative quantification is a normal procedure in QPCR: multiple mathematical approaches exist producing absolute quantities of a target gene based on relative fold differences to a reference gene, and E , the amplification efficiency of each gene (Livak 1997, Phaffl 2001, Liu and Saint 2002). Therefore we recommend that brineshrimp addition be used as a method of calibration in a diverse range of studies.

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