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A Robust Method for Effective Metatranscriptomic RNA Extraction from Puerto Rico's Planktonic Communities Using Low-Cost, Easily-Sourced Material

Brent A. Petersen*, Nikolaos V. Schizas**
*(Department of Marine Science, University of Puerto Rico at Mayagüez, Mayagüez, PR 00681, USA
Email: brent.petersen@upr.edu)
** (Department of Marine Science, University of Puerto Rico at Mayagüez, Mayagüez, PR 00681, USA
Email:nikolaos.schizas@upr.edu)

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Abstract:
The application of metatranscriptomics to the study of plankton communities requires a robust method for the commensurate extraction of RNA from all species of the assemblage. Existing protocols for cell lysis have proven inadequate for meeting this need when applied to the dinoflagellate-rich waters of coastal Puerto Rico. Here we outline the use of an airbrush for a novel approach to effective cell wall disruption of plankton via extreme exposure to the third and fourth derivatives of position. We include procedures for pre-lysis handling of coastal phytoplankton, including collection of samples from embayments of southwestern Puerto Rico.

Keywords **—Plankton, RNA Extraction, Metatranscriptomics.**

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I. INTRODUCTION

Key to understanding Puerto Rico's coastal ecology is an in-depth knowledge of its trophic underpinnings. Marine food webs are supported by primary producers, such as phytoplankton and their microbial grazers [1-3]. Likewise, a fishery's capacity for commercial exploitation is directly correlated to its primary production [4]. The Puerto Rico and US Virgin Islands (PRVI) fishery is sustained by energy from phytoplankton, algae, seagrasses, and the detrital input of mangrove trees. The contribution from phytoplankters in tropical mangrove lagoons can be up to 8.3 g C/m^2/day. Some of this energy is transported to the commercial fishery by allochthonous interlopers and pelagic species that begin their ontogenetic journey amongst the mangroves [5]. The diversity, abundance, and metabolic processes of coastal phytoplankters and associated microbes should be studied throughout the year to better understand the ecosystem and monitor its health and to enlighten decisions on local fishery management.

The pairing of next generation RNA sequencing with advanced gene assembling algorithms has yielded the opportunity of a metatranscriptomic assessment of this foundational portion of the biome [6]. With this powerful tool, the planktonic assemblages of a region can be surveyed, and their functional genomic activities monitored.

A meaningful metatranscriptome analysis requires the cDNA of high-quality mRNA as its input [7]. The attainment of this RNA is comprised of six major tasks: sample collection, sample concentration, cell lysis, intact RNA extraction, RNA purification, and mRNA enrichment. When the transcripts to be studied reside within an environmentally derived planktonic amalgam, there are particular challenges inherent to each step of this process.

The most problematic step in attaining a planktonic metatranscriptome is cell wall disruption. This act is intentionally destructive and should be implemented at the minimum magnitude required to achieve its purpose and aim for discriminant breakage. Its potential to degrade RNA should be

considered with the intent to moderate collateral damage to the RNA being unlocked. The highly labile nature of RNA necessitates that the process be accomplished quickly [8]. While many methods have been developed to lyse the cells of various organisms, they can have limited efficacy in breaching the refractory cell walls of certain planktonic species, particularly dinoflagellates. The genes of these organisms, though likely abundant in the sampled water, can be underrepresented in or entirely absent from the metatranscriptomic analysis if their cells survive the extraction process intact.

There are two broad classes of cell lysing techniques, which can be employed individually or in tandem: chemical and physical. The types of chemical attacks that can be directed at the cell are diverse. Enzymes, such as lysozyme, can be used to degrade the cell wall through the hydrolysis of glycosidic bonds [9-11]. Solvation of the phospholipid bilayer using ionic liquids, dimethyl sulphoxide, or alcohols; such as ethanol, isopropanol, and phenol, can compromise the cell wall [12-14]. Detergents, such as sodium dodecyl sulphate (SDS), may be used to solubilize cell wall proteins and lipids with the added benefit of inactivating RNAses [15-17]. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), can destabilize cell membranes by sequestering the Ca^{++} ions that crosslink their pectin component [18].

A variety of physical forces can be brought to bear against the cell wall. Freeze-thaw cycling can macerate the cell wall via ice crystal growth or burst it through osmotic pressure [19-20]. There are diverse homogenization schemes which may involve bead beating or shear stress and tension administered by blades or passage of the cells through narrow channels [21-24]. In bead beating, the cell wall can be smashed and abraded through by the collision and grinding action of glass beads. The cell membrane can also be punctured with a pulsed electric field [25].

Several artifices within the physical cell disruption arsenal can be subcategorized as the application of pressure or its fluctuation. These include cavitation, French pressing, and osmotic shock. Cavitation can be induced through ultrasonic irradiation or nitrogen decompression [26-27]. Cavitation can rapidly spike the temperature at the nanoscale by thousands of degrees centigrade and swing the pressure by nearly 100 atmospheres. These extreme conditions can decompose phenol, which is a component of many lysing solutions, and damage the RNA through depolymerization [28-29]. In a French press, cell suspensions are subjected to high pressure followed by rapid decompression and shear forces as they are released through a narrow orifice [30]. Internal pressure can be raised, and cells can be burst by osmotic shock when they are immersed in hypotonic solutions [31].

While the above-mentioned methods are employed routinely to successfully extract RNA, they are inadequate for the lysis of some marine phytoplankton cells. In this protocol, we outline a novel approach to cell lysis that was developed for the dinoflagellate-rich plankton community found in the Bioluminescent Bay in La Parguera Natural Reserve at Lajas, Puerto Rico and lab-grown dinoflagellate monocultures. Rapidly applying rapid acceleration to these cells will subject them to the violent effects of jerk and jounce, the third and fourth derivatives of position [32-35]. Dinoflagellate cells slurried in TRIzol™ Reagent can be suddenly accelerated using the Venturi effect [36]. This process can be carried out practically and economically with an airbrush. As the cells transit the nozzle, they will encounter the Venturi effect with its attendant jerk and jounce resulting in their breakage. This can be a stand-alone treatment or combined with other lysis strategies. Major steps are visually summarized in a process flow diagram (Fig. 1). Illumina next generation sequencing reads of RNA extracted via this method from six field-collected plankton amalgams and one laboratory grown dinoflagellate monoculture were analyzedusing fastp to generate the quality-profiling information found in table 1 [37].

II. METHODS

A. Field Collection of Sample

It may be necessary to process large volumes of seawater to acquire sufficient material for RNA harvesting. For this reason, field collection is best accomplished by propelling seawater onto a 25µm-

stainless-steel mesh using a submersible bilge pump, such as the Seachoice® 1000, suspended at the desired depth. While flow rate onto the mesh can be controlled by pump speed, this is not ideal since motile plankton can avoid capture through rheotaxis or in response to visual cues if the apprehension rate is insufficient [38-39]. It is better to run the pump at full speed and divert excess flow from the mesh using a valved manifold.

Once plankton collection is complete, distilled water should be used to wash the sample from the mesh and into a 50mL-conical tube. This will minimize salt carryover, which may confound subsequent processing steps. The filled tubes should be immersed in liquid nitrogen immediately to freeze the samples, which must remain frozen during transport to the laboratory for further processing. In addition to their intended purposes, the distilled water and liquid nitrogen may fortuitously contribute to lysis through osmotic shock and freezing, respectively.

B. Plankton Pelleting

Prior to breaking the planktonic cell walls for RNA extraction, the samples need to be concentrated in the form of a pellet. This is accomplished through centrifugation. Many planktonic species, including dinoflagellates, can display neutral or positive buoyancy [40], which necessitates special measures to achieve pelletization. The aqueous plankton suspension should be diluted with equal parts of cold isopropanol and mixed well to lower the density of the liquid phase before centrifugation. The addition of isopropanol may have the ancillary benefit of weakening the cell walls of the phytoplankton. Afterward, the suspension must be centrifuged at 11,100 RCF for 10 minutes at 4°C to yield a pellet.

C. Cell Lysis and RNA Extraction

In a 15mL-conical tube, add 5mL of TRIzol™ Reagent and 0.25cc of the previously formed plankton pellet. The volume ratio of the pellet to TRIzol™ solution may vary depending on the desired viscosity. Vortex the tube to thoroughly disperse the plankton in the TRIzol™ solution and then allow to incubate at room temperature for five minutes.

After incubation, transfer the TRIzol™/plankton slurry into the cup of a gravity feed airbrush. Use an airbrush with a nozzle diameter between 0.2mm and 0.5mm, such as the Timbertech model ABPST01. The airbrush should be set up to dispense disrupted cells into a vented collection chamber. A suitable collection chamber can be made by piercing the side of a plastic condiment squeeze bottle and inserting the airbrush nozzle, making sure to form a tight seal between the nozzle and the entry port. Set the compressor to supply air at a pressure of approximately 4 atmospheres to the airbrush. Spray the entire TRIzol™/cell slurry into the collection chamber. As the cells enter the vena contracta of the airbrush nozzle, they will be jerked and jounced by the sudden acceleration. Additionally, the leading edge of each cell will experience stronger accelerative force than its trailing edge, which could result in tidal disruption of the cell wall. The turbulent flow will also have a jet milling effect on the cells as they collide with one another.

Transfer 1mL-aliquots of airbrush-processed slurry from the collection chamber into 2mL-microcentrifuge tubes treated with Sigmacote® containing 0.3g of 150µm glass beads and 0.5g of 500µm glass beads and vortex for five seconds. Add 200 µL of chloroform into each tube and vortex for 15 seconds. Incubate the tubes for three minutes at room temperature. Centrifuge at 12,000 RCF for 15 minutes after the incubation period. The RNA will be in the top, clear layer. Transfer this layer to a clean and sterile microcentrifuge tube and proceed to purify the extracted RNA.

D. RNA Cleanup and mRNA Enrichment

RNA purification should be carried out using a commercially available kit, such as the NorgenBiotek® 23600 RNA Clean-Up and Concentration Kit, following manufacturer's instructions. While poly-A tail selection is often used for mRNA enrichment due to its low cost and simplicity, it is inappropriate for a marine plankton metatranscriptome. It is recommended to use rRNA depletion so as not to miss bacterial and

plastid mRNA, as well as mRNA fragments lacking the poly-A tail and to avoid 3' end bias [41].

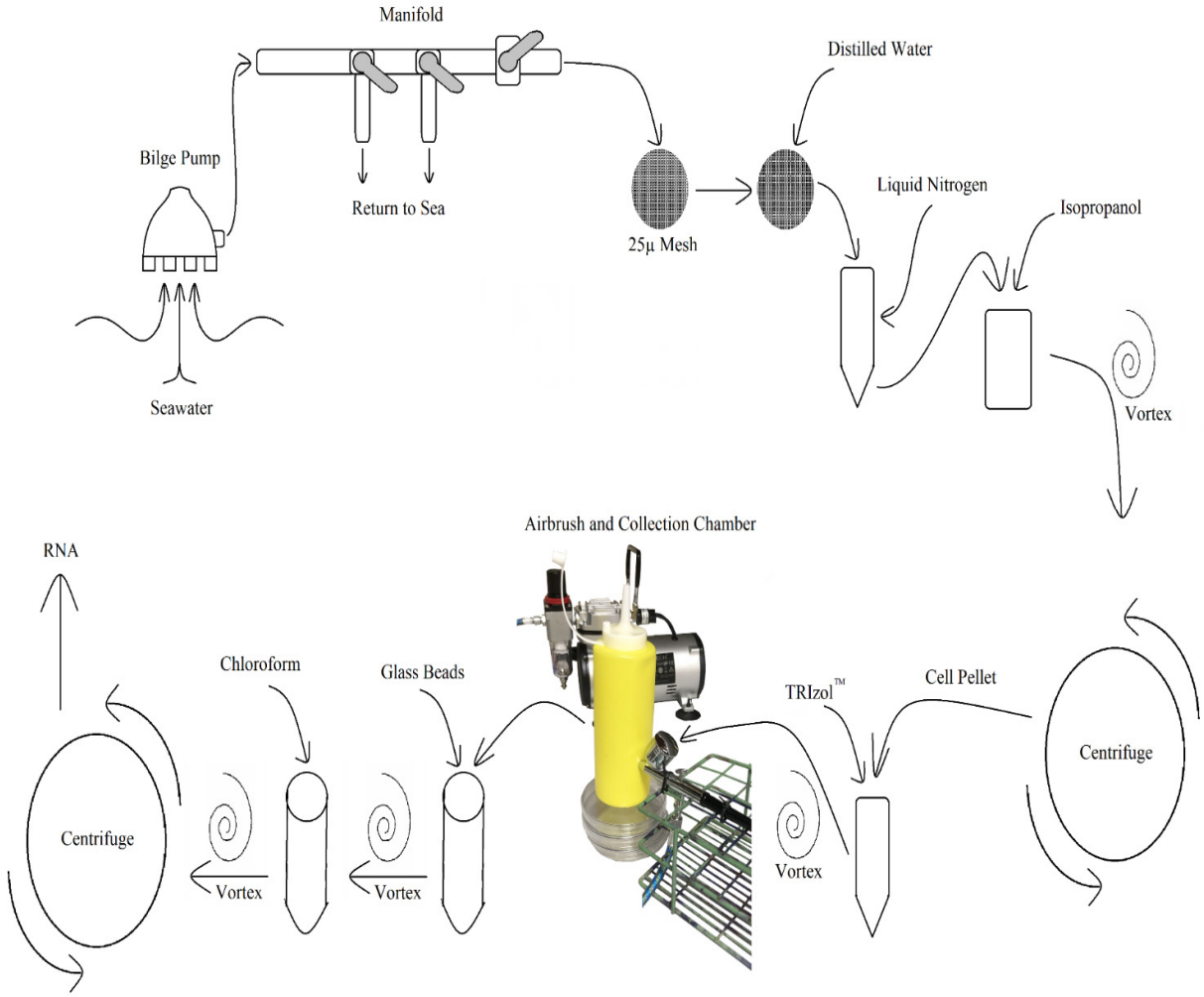


Fig. 1. Process flow diagram.

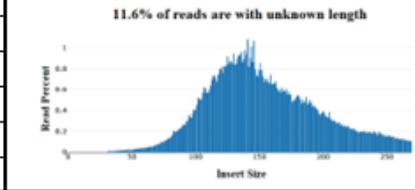
Quality-Profiling Results			Insert Size Distribution
Mangrove Lagoon Metatranscriptome 1	mean length	150bp, 150bp	
	total reads	682.950690 M	
	total bases	102.442604 G	
	Q20 bases	99.078626 G (96.716232%)	
	Q30 bases	93.908969 G (91.669838%)	
	GC content	44.72%	
Mangrove Lagoon Metatranscriptome 2	mean length	150bp, 150bp	
	total reads	568.108538 M	
	total bases	85.216281 G	
	Q20 bases	82.304900 G (96.583539%)	
	Q30 bases	77.996021 G (91.527136%)	
	GC content	43.86%	
Mangrove Lagoon Metatranscriptome 3	mean length	150bp, 150bp	
	total reads	648.601038 M	
	total bases	97.290156 G	
	Q20 bases	93.555243 G (96.161058%)	
	Q30 bases	88.387936 G (90.849824%)	
	GC content	45.50%	
Mangrove Lagoon Metatranscriptome 4	mean length	150bp, 150bp	
	total reads	689.990672 M	
	total bases	103.498601 G	
	Q20 bases	99.957749 G (96.578841%)	
	Q30 bases	94.564427 G (91.367832%)	
	GC content	48.82%	
Mangrove Lagoon Metatranscriptome 5	mean length	150bp, 150bp	
	total reads	275.751284 M	
	total bases	41.362693 G	
	Q20 bases	40.364023 G (97.585578%)	
	Q30 bases	38.590152 G (93.297002%)	
	GC content	41.91%	
Mangrove Lagoon Metatranscriptome 6	mean length	150bp, 150bp	
	total reads	631.840938 M	
	total bases	94.776141 G	
	Q20 bases	91.265895 G (96.296277%)	
	Q30 bases	86.460210 G (91.225713%)	
	GC content	46.61%	
Dinoflagellate Monoculture Transcriptome	mean length	150bp, 150bp	
	total reads	660.268310 M	
	total bases	99.040246 G	
	Q20 bases	95.982968 G (96.913095%)	
	Q30 bases	90.972496 G (91.854068%)	
	GC content	46.73%	

Table 1. Read quality-profiles of RNA extracted via this method from six field-collected plankton amalgams and one laboratory grown dinoflagellate monoculture.

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