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Effects of sample preservation on marine microbial diversity analysis

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ABSTRACT

Three replicate seawater samples were collected on three different days, filtered immediately and preserved with one of two guanidinium thiocyanate-based preservatives (DNAzol™ or RNA Lysis Buffer™ plus β-mercaptoethanol (RLA+)) and were kept frozen while being shipped to a lab. In parallel, a carboy of seawater was collected on each of the three days and maintained at ambient temperature while being shipped to a lab. Upon receipt the samples were filtered and treated in the same manner as for immediate preservation. Significantly more DNA was obtained from samples immediately preserved with DNAzol than the corresponding shipped samples for 2 of the 3 days. More DNA was extracted from DNAzol preserved samples but more RNA was obtained from RLA+ preserved samples. A protocol was designed to extract both DNA and RNA from split samples preserved with RLA+ and cDNA was synthesized from the RNA. Three high-throughput 16S rRNA gene libraries were constructed, one from DNA preserved with DNAzol, one from DNA preserved with RLA+ and one from cDNA (RLA+ preserved). Greater alpha diversity was found for libraries constructed from immediately preserved vs. shipped samples for both preservation types, with immediate preservation with DNAzol obtaining the highest level of diversity. Libraries constructed from immediately preserved (RLA+) DNA had greater alpha diversity than libraries constructed from shipped preserved (RLA+) DNA or cDNA. Unifrac measures of beta diversity showed clearer separation of sample types and a greater % variance explained for weighted than for unweighted principal coordinate analysis (PCoA) plots, indicating sample types varied more in their relative abundance of taxa than the presence/absence of particular taxa. We recommend immediate preservation of seawater samples, with DNAzol as the preferred preservative if quantification via qPCR will be performed or the highest alpha diversity is desired but preservation with RLA+ if RNA will be extracted.

1. Introduction

Ideally, samples should be processed quickly on-site to extract nucleic acids for analysis of microbial community diversity. However, on-site processing is often not feasible for remote or poorly equipped sites and samples are frequently shipped before extracting nucleic acids. Samples held without stabilization by freezing and/or chemical preservation show changes in microbial community composition (Rissanen et al., 2010; McCarthy et al., 2015; Mäki et al., 2017; De Paula et al., 2018). Therefore, it is critical to evaluate stabilization protocols for samples that will be used for molecular analysis. This study compares the effect on the microbial community diversity of seawater samples that are filtered and stabilized by freezing and chemical preservatives versus seawater that is shipped to an inland lab before being filtered and stabilized.

Our previous studies have focused on preservation and handling of samples from oil and gas processing facilities (Duncan et al., 2009; Oldham et al., 2012; De Paula et al., 2018). These microbial communities have low diversity (Bonifay et al., 2017; Cluff et al., 2014; Marks et al., 2017) and are often from anaerobic, hot environments (Duncan et al., 2009; Magot et al., 2000). We find such samples undergo marked changes in microbial community composition if shipped without chemical preservatives (De Paula et al., 2018, and unpublished). In contrast, near-surface seawater microbial communities are highly diverse (Biers et al., 2009; Sunagawa et al., 2015; Yilmaz et al., 2016), supplied with oxygen and sunlight, and experience more moderate temperatures. Seawater microbial communities are quite different from previously tested oil facility communities and provide a greater scope to detect alterations to a highly diverse community.

The variables in this study included samples taken on three consecutive days, two different preservation reagents containing guanidinium thiocyanate, two lysis regimes, and two extraction protocols. Both extraction protocols use the Maxwell®16 instrument (Promega Corp., Madison, WI). We previously compared the use of three different

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Table 1
Sample coding and timeline. Each sample consisted of 3 L filtered seawater. "Immediate" (e.g. I**) samples were preserved after filtering either with DNAzol (*D*) or RLA + (*R*) and freezing. Shipped samples were seawater shipped in a carboy (A, B, C) and filtered upon arrival at OU, then preserved with either DNAzol or RLA + and freezing. DNA was extracted from the samples preserved with DNAzol as described in 2.4. Both DNA and RNA were extracted from the samples preserved with RLA + as described in 2.5. cDNA libraries were synthesized from RNA as described in 2.6.

Immediate samples	DNAzol	RLA +	Shipped samples	DNAzol	RLA +
Day 1	ID1	IR1DNA, IR1cDNA	Carboy A	AD1	AR1DNA, AR1cDNA
	ID2	IR2DNA, IR2cDNA		AD2	AR2DNA, AR2cDNA
	ID3	IR3DNA, IR3cDNA		AD3	AR3DNA, AR3cDNA
Day 2	ID4	IR4DNA, IR4cDNA	Carboy B	BD4	BR4DNA, BR4cDNA
	ID5	IR5DNA, IR5cDNA		BD5	BR5DNA, BR5cDNA
	ID6	IR6DNA, IR6cDNA		BD6	BR6DNA, BR6cDNA
Day 3	ID7	IR7DNA, IR7cDNA	Carboy C	CD7	CR7DNA, CR7cDNA
	ID8	IR8DNA, IR8cDNA		CD8	CR8DNA, CR8cDNA
	ID9	IR9DNA, IR9cDNA		CD9	CR9DNA, CR9cDNA

Day 1 sample: collected January 15th.
Day 2 sample: collected January 16th.
Day 3 sample: collected January 17th.
Carboy A: received and filtered January 18th.
Carboy B: received and filtered January 22nd.
Carboy C: received January 22nd, filtered January 23rd.

nucleic acid extraction kits for extracting DNA from seawater and oil-based slurry (Oldham et al., 2012). Of the three, the Maxwell®16 automated instrument used in combination with the Maxwell® 16 Tissue LEV Total RNA Purification Kit (AS1220, Promega Corp.) provided good recovery of DNA and the lowest variation in DNA quantity among replicate subsamples. We have used the Maxwell®16 instrument and AS1220 kit to extract DNA from a variety of samples including seawater (Aktas et al., 2012; Bonifay et al., 2017; Duncan et al., 2017; De Paula et al., 2018; Lee et al., 2012; Marks et al., 2017, among others). In this study we evaluated the use of the Maxwell instrument to extract RNA from seawater.

The metrics assessed were the quantification of extracted DNA and RNA and estimation of alpha (within community) and beta (between communities) diversity. Rarefaction curves (graphs of species richness versus the number of sequences analyzed, Gotelli and Colwell, 2011) were used to measure alpha diversity in each of the 16S rRNA gene libraries. Beta diversity was estimated by Unifrac measures (Lozupone et al., 2007), which incorporate information from phylogenetic trees to determine the dissimilarity between communities. In unweighted unifrac, the distance between two communities is calculated based on the presence or absence of taxa in the communities, whereas in weighted unifrac, the distance measure also incorporates the relative abundance of the observed taxa. Principal coordinates analysis (PCoA) is used to plot the distance matrix generated by multiple samples on the first three principal coordinates axes to visualize how different samples cluster.

Recommendations are made for the preservation and extraction of DNA alone versus both DNA and RNA. Reproducible changes in the microbial community occurred in shipped samples and reflect primarily differences in the relative abundance of specific taxa rather than the loss of taxa.

2. Materials and methods

2.1. Sample collection and processing

Seawater was obtained at the Key West Naval Research Laboratory from a polyvinyl chloride (PVC) pipe submerged about 3 m below the water surface adjacent to the laboratory. The pipe was capped at its base in the sea and had inlet holes drilled every 0.3 m from 0.3 m from the base up to 1.2 m from the base. The pipe terminated in a spigot on shore. Immediately prior to sample collection for the day, the spigot was turned on and allowed to flush for 2 min, the carboy (Nalgene™, low density polyethylene, 50 L, with spigots) was rinsed three times with seawater, then filled, brought into the laboratory and the contents filtered (1 L Nalgene™ filter units, 0.2 μm polythersulfone (PES) filters). Each filter unit was filled to 1 L, vacuum applied until all liquid passed through, the receiver container was detached and the filtered seawater discarded. The unit was reassembled, and a second liter of seawater filtered, and repeated again until a total of 3 L seawater was filtered per sample. The filter was promptly removed with the aid of sterile (autoclaved) tweezers and a sterile sharp spatula, placed in a sterile 50 mL centrifuge tube containing 2 mL either of DNAzol® (DN127, Molecular Research Center, Cincinnati, OH),) or RNA Lysis Buffer (Promega Corporation, Madison, WI) with β-mercaptoethanol added to 1% final volume (RLA +), mixed to ensure the preservative coated the filter, and placed on dry ice. The seawater was mixed between samples by swirling the carboy.

All samples designated for RNA extraction were filtered first, followed by those for DNA. The time required for two people to process the seawater ranged from 21 to 40 min from filling the carboy until all RNA samples were filtered and placed on dry ice, with an additional 14 to 34 min to process the DNA samples and place them on dry ice. Additional samples were filtered and preserved as above for testing lysis and extraction protocols. After each day's samples were processed, the tubes containing the filters with preservative fluids were placed in a -20 °C freezer on dry ice until shipped to the University of Oklahoma (OU, Norman, OK) via Federal Express in a styrofoam container with dry ice. These are the samples designated as "Immediate", to indicate they were processed immediately after collection. Once received at OU, the tubes were placed in a -80 °C freezer.

The carboy used that day was emptied, refilled, covered with a black plastic bag to exclude light, placed in a box, and shipped via Federal Express. Once received at OU (3–6 days), filtering and subsequent processing took place as described above except the tubes were briefly placed on wet ice and frozen at -80 °C. The samples processed at OU are designated as "Shipped". Samples were collected on three consecutive days, January 15-17th, 2013. Table 1 summarizes when the samples were collected, when received after shipping, the preservation method, and whether DNA alone or both DNA and RNA were extracted from the sample.

2.2. Comparison of preservation reagents: extraction on site

Two samples of 375 mL filtered seawater, one preserved with DNAzol and one with RLA + were processed on the same day they were filtered using the Maxwell® 16 Tissue LEV Total RNA Purification Kit (AS1220, Promega Corp.). Five hundred microliters of sample fluid from the 50 mL centrifuge tube containing the filter and preservative

was combined with 250 µL RBD for each. The DNAzol-preserved sample in addition received 250 µL RLA and the RLA-preserved sample received 250 µL nuclease-free water. The mixtures were loaded into the 1st well of the AS1220 kit cartridge and run according to the FFPE/cell DNA program as described previously (Oldham et al., 2012). After extraction three subsamples of 5 µL each were quantified by fluorometry, using the Qubit® 2.0 Fluorometer (Molecular Probes, Inc., Eugene, OR) Qubit™ dsDNA HS Assay Kit for DNA quantification and the Qubit™ RNA Assay Kit for RNA quantification.

2.3. Comparison of lysis and extraction protocols

A sample filtered and preserved with DNAzol was subdivided and used to compare two lysis reagents, one from the AS1220 Tissue RNA kit and one from the Maxwell®16 LEV simplyRNA Blood Kit (AS1310, Promega Corp.), and two extraction kits (Tissue RNA and simplyRNA Blood). The 50 mL tube containing the filter (tube 1) was retrieved from the −80 °C freezer, briefly thawed, the 2 mL fluid removed to a fresh 50 mL tube (tube 2), 1 mL nuclease-free water was added to tube 1 and contents vortexed briefly at highest speed. The fluid in tube 1 was added to the fluid in tube 2, vortexed to mix, and 1.3 mL was added to each of two screwcapped 2 mL tubes (A, B). Tube A received reagents specific to the Tissue RNA kit, 225 µL each RLA and RNA Dilution Buffer. Tube B received reagents specific to the simplyRNA Blood kit, 225 µL 1-thioglyceryl homogenization solution and 200 µL Lysis buffer. Each tube also received 50 µL proteinase K from the simplyRNA Blood kit, was mixed, incubated 10 min at room temperature, as per the protocol for the simplyRNA Blood kit. The contents of each screwcapped tube was divided, with 800 µL processed in the simplyRNA Blood cartridge using the simplyRNA Blood Maxwell16 program, and 800 µL processed in the Tissue RNA cartridge, using the FFPE/cell DNA program, as described in Oldham et al. (2012, the protocol does not use DNase I). The Qubit ds DNA High Sensitivity and the Qubit RNA reagents were used to quantify double-stranded DNA and RNA, respectively, on triplicate 3 µL samples from each of the 4 combinations of lysis and extraction protocols.

2.4. DNA extraction from DNAzol-treated filters

The protocol for DNA extraction from DNAzol-treated filters was optimized for yield as follows: The 50 mL tube containing the filter was retrieved from the −80 °C freezer, briefly thawed, and 1 mL nuclease-free water and 10 µL proteinase K added, mixed, incubated for 1 h at 37 °C. Reagents from the Tissue RNA kit were added (500 µL each RLA and RDB) and one tube of Lysing Matrix E beads (MP Biomedicals, Santa Anna, CA) vortexed briefly. The 50 mL tubes were inserted into a 50 mL tube vortex adapter (MOBIO, Carlsbad, CA) and shaken at highest speed for 2 min. The tubes were spun at 6000 x g for 2 min to collect the shredded filter pieces and beads at the bottom, the supernatant was removed and divided among the first wells of 4 AS1220 cartridges. The Maxwell16 instrument was programed for the FFPE/cell DNA program as per Oldham et al. (2012). After extraction, the elution tubes were spun at 10000 x g for 1 min, the fluid contents of the four elution tubes removed and pooled, avoiding any small pellets of ground beads or other debris, and the total volume brought to 400 µL using nuclease-free water. All centrifugation steps were performed at room temperature.

2.5. DNA and RNA extraction from RLA-preserved filters

The protocol for parallel DNA and RNA extraction from RLA-treated filters was performed after optimization as follows. The protocol used the Maxwell simplyRNA Blood kit components (AS1310). The 50 mL tube containing the filter was retrieved from the -80 °C freezer, briefly thawed, and 1 mL nuclease-free water, 224 µL 1-thioglyceryl homogenization solution, 200 µL Lysis buffer and 50 µL proteinase K added, vortexed, and incubated for 15 min at 37 °C. The tubes were spun at 6000 ×*g* for 2 min to collect the filter pieces at the bottom, the supernatant was removed and divided among the first wells of 4 simplyRNA Blood cartridges as follows: 200 µL each into 3 cartridges for RNA extraction, 800 µL into one cartridge for DNA extraction. As per manufacturer's instructions, 10 µL DNase I was added to the 4th well of each of the three cartridges used to extract RNA. RNAsin™ Plus RNase Inhibitor (2 µL, Promega™) was added to the nuclease-free water in the elution tubes that would receive the extracted RNA. The manufacturer's run protocol for simplyRNA Blood used. DNA and RNA concentrations were estimated using Qubit reagents as above.

2.6. cDNA

RNA extracted using the protocol above was treated with TURBO™ DNase (Invitrogen™, Carlsbad, CA, 12 µL RNA, 2 µL DNase) as per the manufacturer's directions before cDNA was amplified using the SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA). PCR amplification using universal 16S primers (519F, 785R, Klindworth et al., 2013) was used to test DNase-treated samples and the negative control after SuperScript treatment for contaminating DNA.

2.7. 16S rRNA gene library preparation and sequencing

DNA or cDNA extracted from the samples was used to create 16S rRNA gene amplicon libraries with unique barcodes. Two libraries were constructed for each sample listed in Table 1. The first amplification (30 cycles) used the M13-519F/785R primer pair (Duncan et al., 2009; Klindworth et al., 2013) in which the forward primer contains the M13 sequence followed by the 16S rRNA-specific sequence. Amplification products were purified from primers by running the entire PCR reaction on an agarose gel and cutting out the 300 bp band with sterile gel-cutting pipette tips (ProCatcher Disposable Tips for Gel Excision, Gel Company, San Francisco, CA) while viewed under blue light illumination (SafeImager™, Invitrogen, Carlsbad, CA). The band was placed in a sterile 1.5 mL microfuge tube with 50 µL of PCR grade water, frozen for at least 2 h before the second amplification step was performed.

The second amplification step (12 cycles) added a unique 12 bp barcode (Hamady et al., 2008) containing the M13 sequence at the 3′ end of the forward primer to each library as previously described (Duncan et al., 2009), using 10 µL of DNA eluted from the cut band as template. The barcoded PCR products were purified using Ampure® XP paramagnetic beads (Beckman Coulter Life Sciences, Indianapolis, IN) following the manufacturer's directions. Each purified product's DNA concentration was quantified using the Qubit® HS ds DNA assay (Life Technologies, Carlsbad, CA, USA), and pooled in equimolar amounts and concentrated using an Amicon® Ultra-0.5 mL 30 K Centrifugal Filter (EMD Millipore, Burlington, MA). Sequencing was performed by the Oklahoma Medical Research Foundation (Oklahoma City, OK) on the Illumina MiSeq platform (PE250 V2 chemistry).

2.8. Sequence analysis-Qiime

Paired-end reads were trimmed and stitched together, and unpaired reads were discarded. Default parameters were used within the QIIME (ver 1.9.0) pipeline to de-multiplex, cluster, and remove chimeras. OTUs were assigned a taxonomy at 97% using the most recent release of the Greengenes database. Sequences were submitted to the NCBI short read archive (SRA) under accession numbers SAMN10594103 and SAMN10594104. The barcodes for each sample are listed in Suppl. Tables 1 and 2.

Table 2
Key West seawater parameters January 2013.

Parameters	Average (STD)
pH	8.69 (0.23)
Conductivity (mS/cm)	53.88 (1.03)
Dissolved Oxygen (mg/L)	7.06 (0.99)
Temperature (°C)	23.83 (1.98)
Salinity (%)	3.55 (0.05)

Average and standard deviation (STD) of 16 readings taken at 15:30 GMT.

3. Results

3.1. Site history and parameters

Several biocorrosion and naval research studies used seawater collected from the Key West Naval Research Laboratory (Lee et al., 2004; Lee and Little, 2007; Lee et al., 2010; Aktas et al., 2012; First et al., 2013; among others). Readings of several parameters important for microbial communities are taken most days. Table 2 summarizes all values for the month during which samples were taken for the current study. Moderate temperatures and levels of dissolved oxygen lead us to expect a mesophilic microbial community, dominated by aerobes/facultative anaerobes. A previous study of samples taken from this same site (Lee and Little, 2007) lists additional chemical analyses and viable bacterial counts (6×10^6 aerobes/mL sample).

3.2. Comparison of preservation chemicals on samples collected and extracted the same day

A set of samples was filtered and nucleic acids extracted that same day (Section 2.2) in order to compare the effect of the preservation chemicals without the added variables of freezing or shipping samples. Replicate filtered samples, differing only by the preservation chemical added to the filter, were compared for the quantity of DNA and RNA extracted using an automated nucleic acid extraction instrument, the Maxwell16. More DNA but less RNA was extracted from the filter preserved with DNAzol than from the filter preserved with RLA+ (Table 3, *t*-test).

3.3. Comparison of two extraction and lysis protocols on filters preserved by DNAzol

A 3 L seawater sample filtered and preserved with DNAzol was subdivided and used to compare two lysis reagents (Maxwell Tissue RNA kit (AS1220) and Maxwell simplyRNA Blood kit (AS1310)) and two extraction kits (Tissue RNA and simplyRNA Blood), in 4 different combinations (Section 2.3). Use of the Tissue kit lysis reagents gave higher concentrations of DNA (Table 4), but only the simplyRNA Blood kit extraction cartridges gave detectable levels of RNA. The optimal combination for DNA yield was using the Tissue kit lysis reagents and cartridge, while for RNA the optimal combination was the simplyRNA Blood kit reagents and cartridge.

Table 3
Comparison of preservation fluids on samples collected and extracted the same day.

Preservative	DNA AVG (STD)*	RNA AVG (STD)*
DNAzol	5.97 (0.30)**	1.73 (0.05)***
RLA+	2.26 (0.07)	2.1 (0.12)

Tissue lysis buffers and extraction kit.
* : ng/μL.
** $P = 0.001$, t-test, 2 tailed, unequal variance.
*** $P = 0.019$, t-test, 2 tailed, unequal variance.

Table 4
Comparison of Extraction and Lysis Protocols (DNAzol-preserved).

Lysis: Extraction	DNA AVG (STD)[a]	RNA AVG (STD)[a]
Tissue: Blood	2.58 (0.85)	1.53 (0)
Tissue: Tissue	4.41 (0.81)	BDL[b]
Blood: Blood	1.93 (0.46)	2.00 (0.17)
Blood: Tissue	0.32 (0.02)	BDL

[a] ng/μL.
[b] Below Detection Limit, < 20 ng/mL.

Table 5
DNA quantified from DNAzol-preserved filters.

Collection Day	Immediate	Shipped	# days before filtering
Day 1	12.2[a](2.07)	3.1 (0.18)	3
Day 2	12.3 (1.59)	2.7 (0.22)	6
Day 3	4.6 (0.54)	3.6 (2.10)	7

Tissue lysis reagents and Tissue cartridges were used. See Materials and Methods (2.4) for details.
[a] Average and (STD) of 3 samples, in ng/μL.

3.4. Comparison of DNA quantified from immediately preserved (DNAzol) versus shipped samples

DNA was extracted from paired samples taken on three different days, and either filtered on site and preserved ("Immediate"), or shipped in a carboy and filtered and preserved after arriving at the laboratory in Oklahoma ("Shipped"). For two of the days, the quantity of DNA extracted from the Immediate samples was considerably higher than the paired Shipped samples (Table 5). The concentration of DNA from the Day 3 Immediate sample was much lower than for Days 1 and 2, and not distinguishable from the concentration in the Shipped samples.

3.5. Comparison of DNA and RNA quantified from immediately preserved (RLA+) versus shipped samples

DNA and RNA were extracted from paired samples taken on three different days.

For these samples, there was no significant difference ($p > 0.5$, 2-tailed *t*-test) in quantity of DNA or RNA when Immediate samples were compared to Shipped (Table 6, cDNA quantities are included for completeness).

Table 6
DNA, RNA and cDNA from RLA + −preserved filters.

Collection day	DNA (ng/μL	RNA (ng/μL)	cDNA (ng/μL)
Day 1	AVG (STD)	AVG (STD)	AVG (STD)
Immediate	2.4[a](0.59)	0.9 (0.02)	104 (22.6)
Shipped	2.6 (0.64)	2.0 (0.64)	114 (4.0)
Day 2			
Immediate	1.5 (0.07)	1.0 (0.16)	232 (30.7)
Shipped	1.6 (0.24)	1.4 (0.15)	244 (40.7)
Day 3			
Immediate	2.0 (0.89)	1.1 (0.12)	167 (76.5)
Shipped	1.9 (0.66)	1.8 (0.76)	231 (43.1)

DNA was extracted from 800 μL filter supernatant, RNA was extracted from 200 μL filter supernatant using the simplyRNA Blood™ lysis reagents and simplyRNA Blood™ cartridges. See Materials and Methods (2.5) for details.
[a] Average and (STD) of 3 samples, in ng/μL.

3.6. Overview of key West seawater microbial communities (16S rRNA gene libraries)

There were 36 libraries representing the DNAzol-treated samples. A total of 521,668 sequences were clustered into 84,172 OTUs. The number of OTUs per library ranged from 5726 to 39,376. The 72 libraries representing the DNA and cDNA samples from the RLA+ treatment had a total of 882,165 sequences clustered into 68,858 OTUs.

These communities are highly diverse, as expected for subtropical seawater. Genus level diversity for DNAzol and RLA+ preserved samples is shown in Suppl. Figs. 1 and 2. Dominant taxa at the Class level for all samples are Gammaproteobacteria (15–20%), Alphaproteobacteria (20–26%), and Flavobacteria (10–20%,Suppl. Figs. 3 and 4). Shipped samples, especially from Day1, have higher relative abundance of *Roseibacillus* (Verrucomicrobiae) while immediately preserved samples have a somewhat higher relative abundance of chloroplasts and Marine Group II (Thermoplasmata, Archaea, Suppl. Fig. 5). However, differences between communities are subtle and reflect changes in relative abundance of many taxa.

3.7. Microbial community diversity: alpha diversity (Rarefaction)

3.7.1. DNAzol-preserved samples

Rarefaction analysis of libraries from samples preserved with DNAzol showed the Immediate samples to be more diverse than the Shipped samples (Fig. 1)).

3.7.2. RLA+ preserved samples

Rarefaction curves using Chao1 as a diversity index show libraries constructed from the Immediate days 1,2,3 DNA have greater diversity than the Shipped sample DNA and all cDNA (Fig. 2, chao1 diversity measure). Diversity of Shipped DNA and cDNA samples were not significantly different from one another (95% confidence interval).

3.7.3. Alpha diversity comparison of DNAzol versus RLA+ preserved sample

The numbers of operational taxonomic units (OTUs, at 3% level) observed after 1000 sequences were sampled were higher for samples preserved with DNAzol than those preserved with RLA+. The number of OTUs was in general higher for Immediate samples than for their corresponding Shipped samples (Fig. 3).

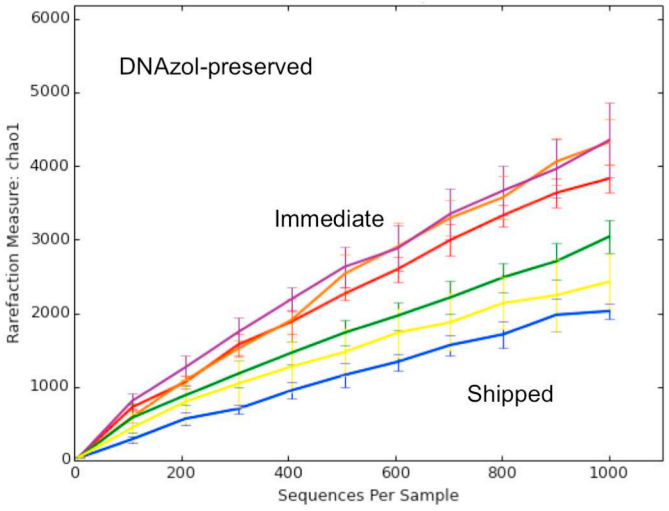

Fig. 1. Rarefaction analysis of 16S rRNA gene libraries from DNAzol-preserved samples. Chao1 was the diversity index (Chao, 1984). Error bars represent 95% confidence intervals.

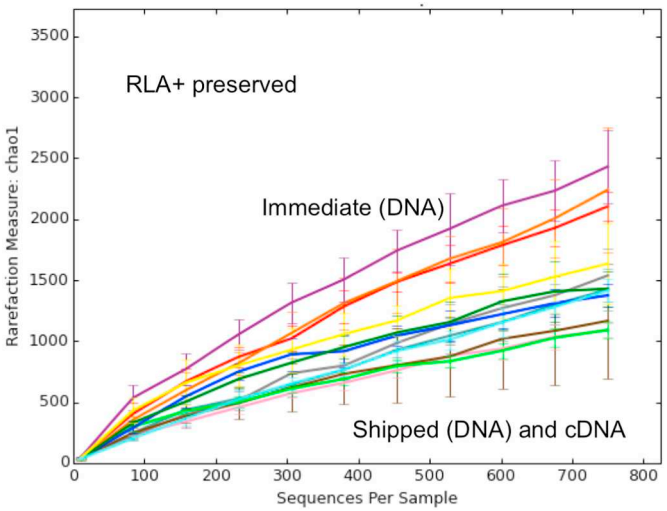

Fig. 2. Rarefaction analysis of 16S rRNA gene libraries from samples preserved with RLA+. Chao1 was the diversity index (Chao, 1984). Error bars represent 95% confidence intervals.

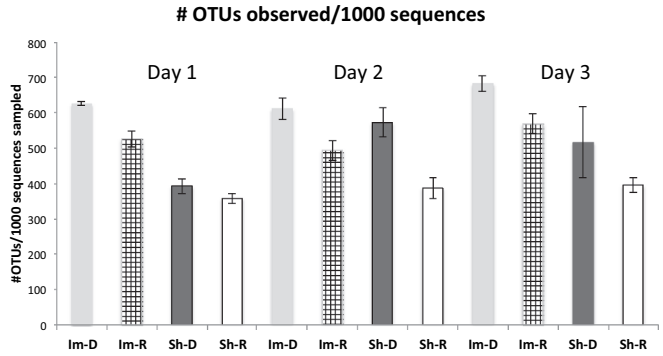

Fig. 3. Rarefaction analysis of # OTUs obtained after 1000 sequences were sampled. Each bar represents the average and 1 STD of 6 libraries.
Im-D: Immediate preservation with DNAzol.
Im-R: Immediate preservation with RLA+.
Sh-D: Shipped, preserved with DNAzol after shipping.
Sh-R: Shipped, preserved with RLA+ after shipping.

3.8. Microbial community diversity: Beta diversity (PCoA plots of unweighted and weighted Unifrac)

3.8.1. DNAzol preserved

Replicates within samples were more strongly clustered in the weighted unifrac PCoA plot than the unweighted plot (Fig. 4), indicating the relative abundance of specific taxa did more to distinguish different samples than did the presence or absence of taxa (Lozupone et al., 2007). As shown in Table 7 (DNAzol—unweighted, weighted) about 50% of the variance is captured by the weighted plot versus 15% by the unweighted.

3.8.2. RLA+ preserved

For both weighted and unweighted unifrac PCoA plots (Fig. 5), PC1 separated cDNA from DNA libraries, while PC2 separated immediately preserved from Shipped samples. More of the variance (approx. 61%) was captured by the weighted plot than by the unweighted (approx. 16%, Table 7), again indicating that the sample groups were mainly distinguished from each other by variation in relative abundance rather than the presence or absence of taxa.

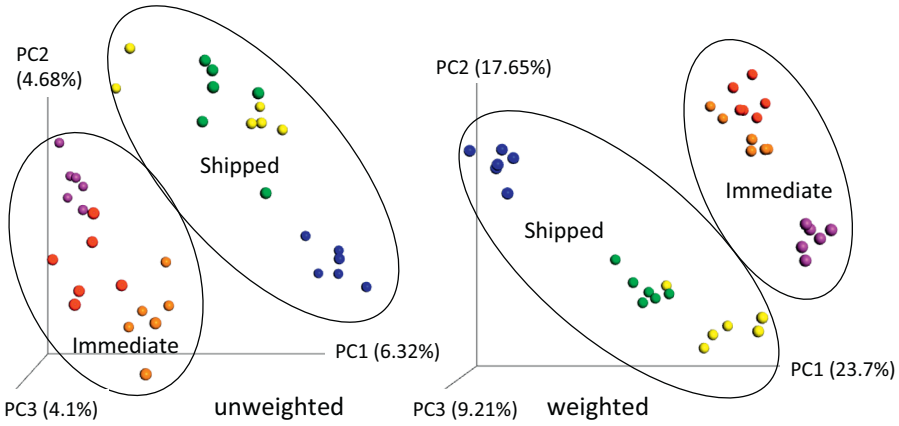

Fig. 4. Unweighted and weighted unifrac PCoA plots of 16S rRNA gene libraries from DNAzol-preserved samples. Immediately preserved samples: red (Day 1), orange (Day 2), purple (Day 3). Shipped samples: blue (Day 1), green (Day 2), yellow (Day 3).

Table 7
% variance captured by individual and summed Unifrac PCoA plots.

Preservative	PC1	PC2	PC3	Sum
DNAzol				
Unweighted PCoA	6.32	4.68	4.1	15.1
Weighted PCoA	23.7	17.65	9.21	50.56
RLA +				
Unweighted PCoA	9.55	4.3	2.38	16.23
Weighted PCoA	40.55	13.76	6.59	60.9

4. Discussion

Sample handling is a vital step in the quest to obtain representative molecular analyses of microbial communities. Effective nucleic acid extraction techniques, primer selection and optimized amplification protocols are also important, but all depend on samples being as little removed from their original state as possible. Ideally, nucleic acids would be extracted from samples on site, but many sites of interest do not have laboratories equipped for molecular analyses or suitably trained technical personnel. Our previous research compared various automated nucleic acid extraction instruments in an effort to bring feasible on-site extraction to sites without molecular laboratories (Oldham et al., 2012).

However, for a number of remote sites the only choices are to ship the sample or to perform limited processing and stabilization. In the current study we compared the effect of a delay in processing/stabilization (e.g. "Shipped") to on-site processing by filtration/preservation by chemical means ("Immediate") for seawater samples collected from Key West, FL. Shipped samples were expected to vary from the Immediate samples, as a previous study using Key West seawater had found marked changes over 120 days in the number of viable aerobes, anaerobes, sulfate-reducing bacteria and acid-producing bacteria regardless of whether the seawater was kept in a sealed container exposed to air or under a variety of conditions promoting an anaerobic environment (Lee et al., 2010). Notably, the number of aerobic viable bacteria in the sealed container exposed to air decreased from 6×10^6/mL sample to 10^2/mL within 30 days. Our study however, was designed to detect even subtle changes in microbial community composition that occurred within a few days, and indeed, reproducible changes were associated with shipped samples.

The small changes seen in the current study with shipped seawater contrast with our experience regarding shipped samples from oil/gas facilities. Typically, if we receive unpreserved samples from thermophilic anaerobic oil/gas facilities, there has been an increase of facultatively anaerobic mesophilic microorganisms (De Paula et al., 2018; and unpublished). Aerobic and facultatively anaerobic mesophilic microorganisms are often introduced into oil/gas facilities during seawater injection or other processes and are distributed throughout the facility. They can remain at low numbers until environmental

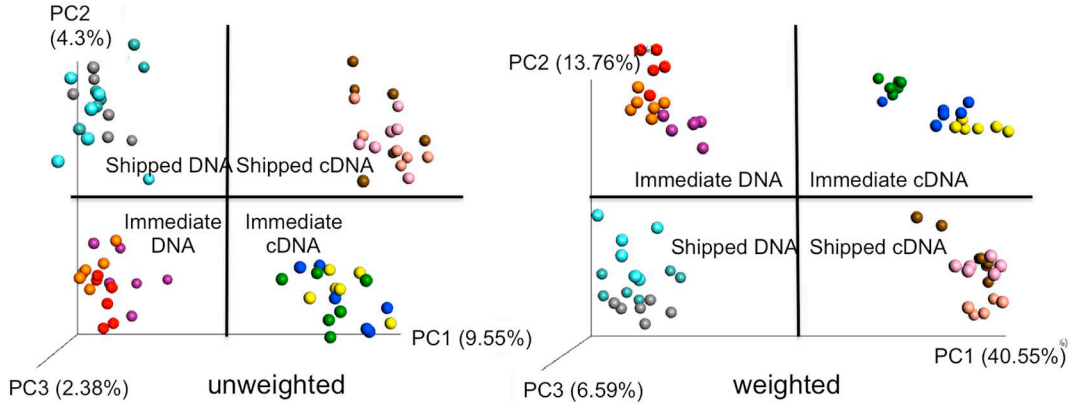

Fig. 5. Unweighted and weighted unifrac PCoA plots of 16S rRNA gene libraries from RLA + preserved samples.
Immediately preserved samples (DNA): red (Day1), orange (Day 2), purple (Day 3). Immediately preserved samples (cDNA): blue (Day 1), green (Day 2), yellow (Day 3).
Shipped samples (DNA): light turquoise (Day1), darker turquoise (Day2), gray (Day3).
Shipped samples (cDNA): pink (Day1), brown (Day2), light orange (Day3).

parameters at a particular site are favorable for their activity and growth (Duncan et al., 2009; Duncan et al., 2009; Shaw et al., 2016).

The choice of chemical preservative made a difference in quantity of nucleic acids obtained and the diversity of 16S rRNA gene libraries, even when samples were immediately placed on dry ice and shipped on dry ice. Our results suggest caution if samples are frozen without chemical preservatives (Haas et al., 2014; Walsh et al., 2016) as there is a risk of the nucleic acids degrading in the period between thawing and completion of extraction. The preservatives used in this study both contained guanidinium thiocyanate (CAS # 593-84-0), often used in nucleic acid extraction protocols due to its great effectiveness in lysing cells and denaturing nucleases (Ausubel et al., 2003). According to its SDS, DNAzol™ (pH 8.5) contained 40–70% guanidinium thiocyanate while the SDS for RLA (pH 7.5) listed 25–50% guanidinium thiocyanate. Other components were proprietary and not described but could have contributed to the differences in preservation seen in this study. RNA extraction protocols often recommend using β-mercaptoethanol in addition to guanidinium thiocyanate as the former is effective in reducing the disulfide bonds in RNases to sulfhydryls (Farrell Jr., 2017). Other studies have found that the chemical preservative used affected the yield of DNA versus RNA as well as the taxa recovered from various sample types (Rissanen et al., 2010; Camacho-Sanchez et al., 2013; McCarthy et al., 2015; Mäki et al., 2017).

Therefore, if nucleic acids cannot be extracted on-site, immediate preservation of the sample is recommended to obtain the highest diversity from marine microbial community samples. Note that our analysis was limited to bacteria and archaea, and did not encompass crustaceans, protists, fungi, and other organisms found in marine waters (First et al., 2013). Our project tested two chemical preservation agents—other agents should be effective if they lyse cells (e.g. prevent growth) and denature nucleases while preventing the degradation of nucleic acids. However, experiments should always be performed to test preservation agents on the specific sample type before sample collection begins.

5. Conclusions

The microbial community composition of shipped seawater has lower diversity than seawater that is filtered on site and preserved.

Chemical preservation with DNAzol is recommended if DNA will be extracted.

Chemical preservation with RLA + β-mercaptoethanol is recommended if RNA will be extracted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.006.

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