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Effects of preservation and storage of microcrustaceans in RNA*later* on RNA and DNA degradation

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Abstract

Methods of preserving nucleic acids are increasingly in demand because of the recent advances in molecular and biochemical approaches to ecology. The RNA storage reagent RNA*later*® was tested as an alternative method to deep-freezing for preserving RNA and DNA in zooplankton. *Artemia* spp. nauplii were used as test organisms. Individual RNA and DNA contents were monitored over a time period of 8 months to evaluate the effects of preservation and storage. Treatments included (1) freezing at –80°C, (2) preservation with RNA*later*, followed by storage at 5°C, and (3) preservation with RNA*later* and storage at room temperature. Freezing at –80°C was the only treatment that did not result in significant change from the initial values in any of the nucleic acids, nor at any time of the experiment. At 5°C, significant RNA degradation was not observed until 8 months after preservation while no significant changes in DNA were detected. In samples stored in RNA*later* without refrigeration, RNA did not exhibit significant decrease for at least 1 month, and DNA for at least 2 months. As RNA and DNA degraded at roughly the same rate, this resulted in little or no changes in RNA:DNA ratios, but within-treatment variability increased strongly. Thus, RNA*later* successfully preserved both RNA and DNA for up to 1 month at room temperature, and up to 4 months at 5°C, providing an alternative to the deep freezing. This method will enable a greater integration of molecular methods in ecological studies.

An increasing number of plankton biologists are using analysis of bulk RNA and DNA for estimating growth and nutritional conditions of zooplankton in field studies and laboratory experiments (e.g., Wagner et al. 1998; Campbell et al. 2001; Vrede et al. 2002; Durbin et al. 2003; Gorokhova 2003; Hansen et al. 2003). Because RNA is closely linked to protein synthesis, its concentration in physiologically active tissues often varies according to the organism's metabolic needs for growth (Alberts et al. 1983). DNA content of somatic cells is roughly constant, so the DNA content of the organism might be used to estimate the total number of cells. Both nucleic acids are involved in the protein synthesis and cellular multiplication required for growth, and therefore, the ratio of RNA to DNA has been regarded as an index of protein synthetic capacity per cell (Bergeron 1997; Buckley et al. 1999). RNA and DNA can be assayed on whole tissue homogenates (Bentle et al. 1981), and over the last several years methods have been developed to enhance precision and accuracy of nucleic acid quantification in small-bodied aquatic organisms, e.g., fluorometric assays in combination with sensitive fluorochromes, use of microplates, and more efficient extraction procedures (Caldarone and Buckley 1991; Nacci et al. 1994; Wagner et al. 1998; Caldarone et al. 2001; Gorokhova and Kyle 2002). These methods provide aquatic ecologists with powerful tools to quantify nucleic acids in as little sample material as a single *Daphnia* egg (Gorokhova and Kyle 2002) that can be used to investigate the links between environmental factors and plankton growth, physiological requirements, and, ultimately, community structure and functioning (Elser et al. 2000).

Sample preparation, preservation, and storage are critical steps in nucleic acid quantification. It is well known that nucleases, and particularly RNases, are very aggressive and can rapidly digest significant amounts of sample RNA and DNA. For this reason, successful extraction requires rapid processing or appropriate preservation of collected samples. Analyzing minute quantities of sample material, such as a single zoo-

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plankton specimen, is a challenging task. The difficulty is increased when samples are obtained away from the laboratory and have to be stored and transported before analysis. Sample preservation for subsequent RNA-DNA analysis is usually done by freezing material in liquid nitrogen. The rapidly frozen (i.e., fast- or snap-frozen) samples are then stored in an ultracold freezer and shipped on dry ice. Traditional methods of "in-tissue" RNA and DNA preservation—cooling on ice or freezing—have been applied to zooplankton (Wagner et al. 1998, 2001; Gorokhova 2003). This is often not feasible in remote fieldwork locations or on cruises. Consequently, lack of convenient preservation methods remains the greatest obstacle for nucleic acid measurements in field studies.

RNA*later*® (Ambion) is a storage reagent that stabilizes and protects cellular RNA in intact unfrozen samples by rapidly penetrating fresh tissue and deactivating nucleases. The reagent is also applicable for DNA preservation (Gorokhova and Kyle 2002; Johnson et al. 2003). According to the general directions of the manufacturer, samples can be stored in RNA*later* for up to 1 day at 37°C, for up to a week at room temperature, for a month or more at 4°C, or stored indefinitely at –20°C without nucleic acid degradation (Ambion 2001). The manufacturer recommendations vary depending on sample material: some organisms (e.g., yeast, Gram-positive bacteria) or tissues (e.g., whole blood) are not suitable for preservation in RNA*later* (Ambion 2001). To date, the reagent has been tested mostly on tissues from vertebrate species and cell cultures. Previous work has demonstrated that RNA*later* with subsequent storage at –20°C performed as good or better as freezing of fresh material at –80°C for maintaining of bulk RNA and DNA in *Daphnia* (Gorokhova and Kyle 2002). The effects of RNA*later* preservation and storage in the absence of freezing facilities are of particular interest. This is a common situation in field studies, and these effects have yet to be explored.

The objective of this study was to evaluate applicability of RNA*later* for fixation and storage without freezing for preserving total RNA and DNA in planktonic microcrustaceans. Newly hatched brine shrimp nauplii (*Artemia*) were used because of their suitable body size along with convenience in obtaining many individuals of the same stage and physiological condition.

Materials and procedures

Sample preparation—The nauplii of *Artemia* spp. (San Francisco Bay Brand) were hatched in a 2-L container supplied with aeration (23‰ to 26‰, 26°C to 28°C) and used within 4 h of hatching. Animals were sorted alive either in the hatching media and subsequently frozen, or on a watch glass filled with 0.2 to 0.3 mL of RNA*later* and subsequently preserved with RNA*later*, using a wide-mouth pipette and an Irwin loop. Each sample consisted of a single individual (Instar I nauplius stage, body length 420 ± 10 µm, mean ± SE, *n* = 20), and 39 samples were randomly assigned among three treatments and controls.

Preservation and storage—Three preservation and storage regimes were applied: (1) deep freezing, in which *Artemia* were placed alive in a nuclease-free 1.5-mL Eppendorf tube and immediately (within 3 min) transferred into a –80°C freezer (–80°C treatment), (2) preservation with RNA*later*, in which *Artemia* were placed in an Eppendorf tube containing 20 µL of RNA*later* (~10 times the animal body volume), and stored at 5°C (RNA*later* + 5°C treatment), and (3) preservation with 20 µL of RNA*later*, followed by storage at room temperature (19 to 22°C) in darkness (RNA*later* + RT treatment). Control samples were obtained by analyzing fresh animals immediately after hatching and sorting (3 replicates; reported as Month 0).

Nucleic acid analysis—Procedures described by Gorokhova and Kyle (2002) were used to quantify RNA and DNA in individual nauplii. Briefly, microplate fluorometric high-range assays using RiboGreen were performed after extraction with N-laurylsarcosine followed by RNase digestion. Measured RNA and DNA concentrations were expressed as ng · ind^{-1}.

Working reagents. RiboGreen™RNA Quantitation Kit (Molecular Probes, cat. nr R11490); RNA (16S and 23S from *Escherichia coli*, Component C of the RiboGreen Kit); DNA (calf thymus, Sigma, cat. nr D-1501); RNase, DNase-free (Q-biogene, cat. nr RNAS0500), working solution 5 µg · mL^{-1}; N-lauroysarcosine (sarcosyl, Sigma, cat. nr L-5125); TE buffer (Q-biogene, cat. nr TE1 × 0001).

Standards. RNA and DNA standard sets were prepared from frozen (–80°C), aliquoted stock. Working solutions were diluted in standard buffer (0.1% w/v sarcosyl in TE buffer) in concentrations ranging 0.01 to 0.12 µg mL^{-1} for RNA, and 0.01 to 0.08 µg mL^{-1} for DNA. The standard sets were aliquoted and stored at –80°C until analysis.

Extraction procedure. When analyzing a sample preserved with RNA*later*, 15 µL of the solution was removed from the tube prior to extraction and discarded. Extraction buffer (1% sarcosyl in TE buffer, 100 µL) was added directly to the samples containing either frozen or RNA*later*-preserved *Artemia*. Samples were shaken at 5°C for 1 h, and then TE buffer was added (900 µL); samples were shaken again for 10 min and centrifuged for 1 min at 9000 rpm.

Fluorometric determinations. Fluorescence measurements were performed using fluorometer FLUOstar Optima (BMG Labtechnologies, microplate reader, filters: 485 nm for excitation and 520 nm for emission) and black solid flat-bottom microplates (COMBO; Labsystems, cat. nr 9502067). The plate was scanned with 0.2 s well measurement time, with 10 measurements per well. Two replicate subsamples for each of the nucleic acids (80 µL · well^{-1}) were measured from each sample following (1) RNA digestion with RNase (5 µL · well^{-1}; 37°C, 30 min) of subsamples designated for DNA determination including DNA standards and (2) RiboGreen addition to all wells (80 µL · well^{-1}, 5 min).

Recovery tests. On two occasions (Month 0 and Month 4), recoveries were determined by spiking 6 samples (3 replicates for RNA and 3 for DNA) of each type of treatment. As a spike, RNA and DNA standard stocks were added to the samples; target concentration of spike was calculated to ensure that spike

plus *Artemia* nucleic acid concentration was within the range of standards. On both occasions, the recoveries were high (97.3% ± 0.9% for RNA and 107.2% ± 1.1% for DNA; n = 18, pooled data), and did not differ between the preservation treatments or the test runs (two-way ANOVAs; RNA: interaction: $F_{1,12}$, $P > 0.4$; treatment: $F_{1,12}$, $P > 0.4$; test run: $F_{2,12}$, $P > 0.7$; DNA: interaction: $F_{1,12}$, $P > 0.8$; treatment: $F_{1,12}$, $P > 0.2$; test run: $F_{2,12}$, $P > 0.9$). Therefore, concentrations in the samples were not corrected for percent recovery of internal standards.

Statistics—The nucleic acid abundance resulting from different preservation and length of storage periods were investigated by performing ANOVAs on the RNA and DNA data (GraphPad Prism 4.01, GraphPad Software). Student Neuman Keuls multiple comparison procedures (SNK test) were carried out to investigate significant differences. Unless specified otherwise, data are presented as arithmetic means along with standard errors. In all cases significance was accepted when $P < 0.05$.

Assessment

Total cellular RNA and DNA were subsequently compared with control samples and among preservation treatments to determine the long-term effects of different preservation techniques. Nucleic acid quantities were determined in 3 randomly chosen samples from each preservation treatment after 1, 2, 4, and 8 months. In total, 75 samples were analyzed (3 for controls, 36 for treatment tests, 36 for recovery tests).

The total yield of bulk RNA and DNA extracted from *Artemia* nauplii was 11 to 77 and 10 to 46 ng · ind^{-1}, respectively. Different preservation techniques resulted in different RNA and DNA yields that varied with time of storage (Fig. 1, Tables 1 and 2). Freezing at –80°C was the only treatment that did not result in significant change from the initial values in any of the nucleic acids, nor at any time during the experiment (Table 1). Although relatively high yields of both RNA and DNA were extracted from frozen animals (Fig. 1A,B), both nucleic acids experienced at least some degradation (decrease in total yield, Table 2), albeit not a statistically significant amount, when compared to control samples analyzed immediately (SNK, $P > 0.05$ in both cases; Table 1). The degradation of RNA as a result of freezing has been indicated previously by the formation of breakdown products of 28S rRNA extracted from snap-frozen autopsy tissues (Ross et al. 1992). Similar effects have also been reported for DNA with both statistically significant (Pesaro et al. 2003) and not significant (Johnson et al. 2003) degradation attributed to freeze-thaw stress.

After 8 months of storage, the RNA content of individuals preserved with RNA*later* and stored at 5°C was significantly decreased compared to that in the controls (Fig. 1A). At no time during the experiment was the DNA yield in this treatment significantly different from either the controls or –80°C samples (Fig. 1B). Thus, for both RNA and DNA, storage in

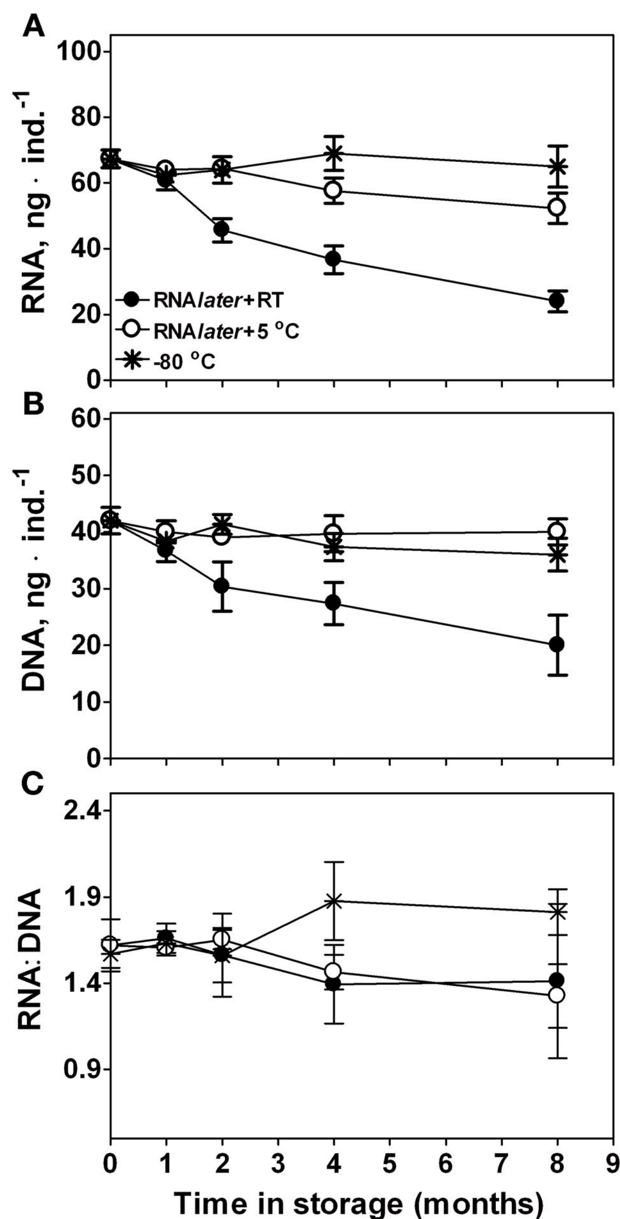

Fig. 1. Time-course of (A) RNA and (B) DNA individual contents (ng · ind.$^{-1}$) and (C) RNA:DNA ratio in *Artemia* nauplii preserved by different methods. Control samples obtained by analyzing fresh animals immediately upon hatching and sorting are reported as 'Month 0'. Data are presented as means and standard deviations.

RNA*later* at 5°C was equivalent to frozen storage at –80°C for at least 4 months after preservation; in both treatments samples exhibited only negligible breakdown (Table 2).

Relative to the frozen and refrigerated samples, those preserved in RNA*later* and stored at room temperature showed slight, although not significant, degradation of both RNA and DNA after 1 month, and continuous decreases up to the end of the trial period (Fig. 1A,B, Table 2). For RNA this decrease becomes significant after 2 months, and for DNA, after 4 months of storage (Table 1). Overall, in this treatment, the

Table 1. ANOVAs and Student Neuman Keuls (SNK) multiple comparison tests of RNA and DNA data on the duration of the storage period (Time: month 0, 1, 2, 4, and 8) and type of preservation (Preservation: RNA*later* + 5°C; RNA*later* + RT; and –80°C)

ANOVA					SNK tests	
Source	df	MS	F	*P*	RNA*later* + 5°C	Month 0 = Month 1 = Month 2 = Month 4 > Month 8
RNA					RNA*later* + RT	Month 0 = Month 1 > Month 2 > Month 4 > Month 8
Interaction	8	231.5	5.893	0.0002	–80°C	Month 0 = Month 1 = Month 2 = Month 4 = Month 8
Preservation	2	1592	40.52	<0.0001	Month 1	RNA*later* + RT = RNA*later* + 5°C = –80°C
Time	4	547.5	13.93	<0.0001	Month 2	RNA*later* + RT < RNA*later* + 5°C = –80°C
DNA					Month 4	RNA*later* + RT < RNA*later* + 5°C = –80°C
Interaction	8	53.12	2.236	0.0528	Month 8	RNA*later* + RT < RNA*later* + 5°C = –80°C
Preservation	2	373.0	15.70	<0.0001	RNA*later* + 5°C	Month 0 = Month 1 = Month 2 = Month 4 = Month 8
Time	4	118.7	4.996	0.0033	RNA*later* + RT	Month 0 = Month 1 = Month 2 > Month 4 = Month 8
					–80°C	Month 0 = Month 1 = Month 2 = Month 4 = Month 8
					Month 1	RNA*later* + RT = RNA*later* + 5°C = –80°C
					Month 2	RNA*later* + RT = RNA*later* + 5°C = –80°C
					Month 4	RNA*later* + RT < RNA*later* + 5°C = –80°C
					Month 8	RNA*later* + RT < RNA*later* + 5°C = –80°C

effect of time in storage on the RNA yield was less variable than that for DNA (least square linear regressions: r^2 = 0.84 and 0.61, respectively). Thus, we found acceptable preservation at room temperature in RNA*later* for at least 1 month for RNA and 2 months for DNA.

Discussion

In remote fieldwork locations, such as on oceanographic cruises, it is often difficult and even impossible to arrange an RNase-free benchtop for molecular investigation of plankton samples, or for cumbersome equipment needed for analytical procedures in the field. Use of liquid nitrogen and/or ultracold freezer is not always possible and is complicated by thawing of material and loss of samples during shipping. RNA*later* was tested to determine how long a microcrustacean specimen can be stored in the absence of freezing without significant RNA and DNA degradation. It appears to provide an alternative to storage at –80°C without the inconvenience associated with freezing.

When stored up to 1 month at room temperature (approximately 20°C) and up to 4 months at 5°C, *Artemia* nauplii preserved in 20 µL of RNAlater solution did not exhibit any significant decrease of either RNA or DNA yields. This exceeds the time usually needed to conduct fieldwork and ship samples to the laboratory for analysis. In addition to obtaining high quantities of nucleic acids, there are other practical advantages of tissue storage in RNA*later*, namely that samples can be preserved immediately upon collection, and sorted later prior to analysis. This flexibility allows easy integration of the collection procedure into complex sampling schedules in field research, when access to freezing and refrigeration is not available.

Interestingly, in all treatments the RNA and DNA values suggested degradation proceeded at roughly same rate during storage (Table 2). The overall RNA:DNA ratio consequently varied little (two-way ANOVA, interaction: $F_{8,30}$, $P > 0.8$; preservation: $F_{2,30}$, $P > 0.2$; time: $F_{4,30}$, $P > 0.9$), although within-treatment variability increased greatly (Fig. 1C, Table 2). As studies generally use RNA:DNA ratios as indices of growth and nutritional conditions, rather than the absolute concentrations (Buckley et al. 1999; Caldarone et al. 2001), samples with

Table 2. Changes in total RNA and DNA yield expressed as percentage of the initial values (i.e., in fresh samples) and within-treatment variability* for each of the preservation methods applied† and the length of storage period‡

	RNA*later* + RT			RNA*later* + 5°C			–80°C		
Time, mo	RNA	DNA	RNA:DNA	RNA	DNA	RNA:DNA	RNA	DNA	RNA:DNA
1	90 (9)	87 (10)	102 (9)	95 (4)	95 (9)	99 (5)	93 (8)	91 (6)	107 (12)
2	68 (13)	72 (25)	96 (27)	96 (5)	93 (5)	102 (6)	95 (11)	98 (7)	96 (17)
4	54 (20)	65 (24)	86 (28)	86 (12)	94 (14)	90 (12)	102 (13)	89 (11)	116 (21)
8	36 (66)	48 (46)	87 (55)	78 (15)	95 (10)	82 (24)	97 (17)	86 (14)	111 (13)

*CV%; values in parentheses.
†RNA*later* + 5°C; RNA*later* + RT; and –80°C.
‡1 to 8 mo.

partially degraded RNA and DNA after 2 to 8 months of storage might still be usable for this purpose. However, the greater variability observed within treatments suggests that increased replication would be needed for a reliable assessment. A large number of replicates can be prohibitively costly and may not be practical for common investigators.

In addition to RNA and DNA, proteins were also found to remain well preserved in RNA*later*, enabling quantification of these compounds from a single sample (Rodrigo et al. 2002). This allows maximum use of available sample material. In zooplankton samples preserved in RNA*later*, a variety of other biomarkers can be assayed, such as the protein:DNA ratio, an index of average cell biomass, and the RNA:protein ratio,which represents the RNA concentration of metabolically active biomass. This could be useful in evaluating zooplankton physiological state as different types of growth that might be best represented by different ratios (Bergeron 1997; Buckley et al. 1999; Wagner et al. 2001).

Comments and recommendations

Because RNA consists of rRNA (75% to 80% of total RNA; Alberts et al. 1983), mRNA (5% to 10%), and tRNA (10% to 15%), the effects of preservation and storage may be different for different classes of molecules. While fluorometric analysis of RNA and DNA provided useful quantitative information, this analysis could not discriminate between different classes of RNA molecules. In particular, as mRNA decays much faster than rRNA or tRNA (Swift et al. 2000), it is reasonable to assume that a larger proportion of mRNA in the preserved tissues would result in lower total RNA recovery. Consequently, the conclusions of this study might not hold true when organisms with grossly different nucleic acid composition—either species-specific or environmentally induced—are collected and preserved. Also, quantitative results are not always indicative of RNA and DNA quality because partially degraded molecules remain detectable by fluorometry (Singer et al. 1997). Thus, quantitative results do not permit critical assessment of the composition and structural integrity of the extracted RNA and DNA molecules that are of particular importance for genetic and molecular studies, and these remain to be tested.

Another area of concern with respect to use of RNA*later* for a whole-body preservation of crustaceans is the presence of an exoskeleton, which must be considered as a diffusion barrier for aqueous sulfate salt solutions such as RNA*later*. The efficiency of this barrier is likely to be low in a case of the small planktonic crustacean with a thin, noncalcified cuticle, as evidenced by successful preservation of nucleic acids in *Artemia* spp. nauplii (this study), *Daphia* (Gorokhova and Kyle 2002), and copepods (Gorokhova and Edlund unpubl. data). However, the exoskeleton structure (and consequently its permeability) in crustaceans may vary between different species and/or ontogenetic stages as well as during a molting cycle of the same individual (Dennell 1960) resulting in different degradation of nucleic acids before the preservative has had an opportunity to penetrate the tissues, especially if during this time samples are not cooled. The efficiency of this preservation and necessity for dissection should be further evaluated if this method is to be applied for preservation of larger pelagic crustaceans with harder and more rigid cuticle (e.g., mysids).

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