Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2013) 35(5): 972–981. First published online June 10, 2013 doi:10.1093/plankt/fbt035

Acetone preservation for zooplankton molecular studies

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Received January 29, 2013; accepted April 8, 2013

Corresponding editor: Roger Harris

Ethanol is one of the most commonly used fixatives for zooplankton samples in molecular studies, despite known problems with this method. Alternative preservation solutions are desired that would reliably preserve DNA over medium to long time scales (months to years). This study tested the efficacy of acetone as a bulk fixative for DNA preservation in marine zooplankton. We used quantitative polymerase chain reaction to measure changes in DNA copy number individual^{-1} over weeks to months in specimens of the planktonic copepod *Parvocalanus crassirostris* that were bulk preserved in (i) acetone, with the fixative changed within 24 h of collection, (ii) acetone, with the fixative not changed and (iii) a standard 95% ethanol bulk fixation protocol. We found that DNA preservation in acetone was comparable to that in ethanol in both short- (30 days) and long-term (4 months) laboratory and field experiments, with modest, but non-significant, declines in DNA copy number individual^{-1} over time. Significant DNA degradation was observed over a 4-month storage period in acetone-preserved samples in which the fixative was not changed immediately following collection. Although acetone provided comparable DNA preservation to ethanol in this study, we also found that precipitates form in the presence of seawater, making identification of copepod specimens difficult in bulk acetone preserved samples. For this reason, we do not recommend acetone as a bulk fixative, but do recommend that it be considered a good alternative for preservation of individual specimens in taxonomic groups that are known to preserve poorly in ethanol (e.g. chaetognaths).

KEYWORDS: preservation; molecular ecology; plankton; nucleic acids; mtCOI; crustaceans

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INTRODUCTION

Molecular tools are increasingly being used in plankton research to address a range of ecological and evolutionary questions. Many of these approaches rely on data from nucleic acids. For example, quantification of bulk RNA and DNA is often used to estimate growth and nutritional condition of animals in the field (e.g. Wagner *et al.*, 1998; Vrede *et al.*, 2002; Gorokhova, 2003; Gusmao and McKinnon, 2011; Koski *et al.*, 2012), while DNA-based fragment analysis and sequencing are the primary tools for population genetics (Rynearson and Armbrust, 2005; Peijnenburg *et al.*, 2006; Goetze, 2011). Quantitative polymerase chain reaction (qPCR) is yielding insights into trophic interactions that were intractable using conventional techniques (e.g. Nejstgaard *et al.*, 2008; Cleary *et al.*, 2012), and the emerging fields of genomics and transcriptomics are addressing a range of questions from organismal responses to stress to genome structure and gene function (e.g. Lenz *et al.*, 2012). All of these fields require reliable preservation of nucleic acids in biological tissues as the first step of the analysis. While cryopreservation is widely recognized to be the preferred preservation method for nucleic acids, deep-freezing of specimens is often difficult or intractable in the field. As a result, a wide range of other chemical treatments have been tested and used for DNA preservation. Dimethyl sulfoxide (DMSO), cetyltrimethyl ammonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), or urea buffers, ethanol, other alcohols (e.g. isopropanol), acetone and RNAlater® (Ambion, Life Technologies) are all used as preservation solutions (Nagy, 2010), with prevalence of use varying across taxonomic groups. For marine invertebrates, the most commonly used preservation methods involve preservation in ethanol solutions (70–100% concentrations), DMSO (or DMSO-NaCl, DMSO-SDS (sodium dodecyl sulfate)), RNAlater® or cryopreservation (Dawson *et al.*, 1998; Bucklin, 2000; Gorokhova, 2005). Ethanol preservation is the most widely used method, as it has long been known to yield high molecular weight (HMW) DNA. However, there also have been many reports of DNA degradation in ethanol-preserved samples, with increasing loss of DNA over long storage times (months–years), at warmer storage temperatures and in samples with higher water content (e.g. Reiss *et al.*, 1995; Holzmann and Pawlowski, 1996; Fukatsu, 1999). In our work, we observed a decrease in DNA copies in planktonic copepods following bulk preservation in ethanol and 41 days of storage at −20°C (as measured in qPCR; Jungbluth *et al.*, in press), and also anecdotally found that ethanol-preserved samples that are several years old (>5 years) cannot be used for more technically demanding applications (microsatellites, next generation sequencing), although mitochondrial (mt) markers can be readily amplified from this material.

Field studies on marine zooplankton present a distinct set of challenges for DNA preservation. First, for many applications, plankton material needs to be preserved in bulk, in order to preserve the DNA as rapidly as possible from the whole plankton community. This constraint means that the preservative solution needs to be relatively affordable, for use in large volumes. The best preservation methods, cryopreservation or preservation in RNAlater®, require sorting of individual live specimens under a microscope, with preservation in small vials (small volumes). Second, when preserving the plankton community in bulk, it is also necessary that the physical integrity of tissues be maintained, such that it is possible to sort and identify animals to species following preservation. A number of preservation methods that have high success in some taxonomic groups result in physical disintegration of the preserved tissue (e.g. Frampton *et al.*, 2008), which makes subsequent identification of organisms impossible. This combination of requirements has resulted in the continued use of ethanol as a bulk fixative for molecular studies of zooplankton, despite known problems with this method. An alternative preservation method is needed for bulk fixation of plankton samples that would preserve DNA integrity over medium to long time scales (months to years).

The objective of this study was to test the efficacy of acetone as a bulk fixative for zooplankton samples that are intended for subsequent use in molecular ecology. Acetone has been reported to be more effective at preserving DNA in samples with high water content (Fukatsu, 1999), which is particularly relevant for the preservation of bulk plankton material. This preservative has been adopted as the method of choice for terrestrial arthropods, and is now widely used to preserve DNA in insects and arachnids as well as their microbial symbionts and parasites (e.g. Braendle *et al.*, 2005; Mancini *et al.*, 2008; Kikuchi *et al.*, 2009; Anbutsu and Fukatsu, 2010; Matsuura *et al.*, 2012). Acetone is also increasingly being used to preserve nucleic acids in crustaceans from diverse environments (Ivey and Santos, 2007; Remerie *et al.*, 2009; Santos and Weese, 2011). In this study, we used qPCR to measure changes in DNA copy number individual^{-1} over time in planktonic copepods preserved using (i) bulk preservation in acetone, (ii) bulk preservation in acetone, with replacement of the acetone within 24 h of initial fixation, and (iii) a standard ethanol preservation protocol. We conducted both short- (1 month) and long-term (4 months) experiments with field-collected samples to quantify changes in DNA copy number individual^{-1} during storage, as well as a short-term

laboratory study in order to control for environmental food effects on DNA copy number. We also examined the level of variation in mtCOI copy number individual^{-1} in replicate samples from within the same plankton tow and between consecutive plankton tows, in order to better resolve sources of variation in mtCOI copy number individual^{-1}. Here, we show that DNA preservation in acetone is broadly comparable, but not superior to, that in ethanol over time scales of weeks to months, when the preservative is changed within 24 h of fixation.

METHOD

A single copepod species, *Parvocalanus crassirostris* (Calanoida: Paracalanidae), was used to test the different preservation methods. This small-bodied calanoid copepod is one of the dominant zooplankton species at our field site in Kāne'ohe Bay, Oahu, Hawaii (Scheinberg, 2004). This study reports results from both field-collected animals and laboratory populations of this species. The laboratory animals were isolated from Kāne'ohe Bay in 2008, and have been maintained in continuous culture on a monoalgal diet of *Isochrysis galbana* (McKinnon *et al.*, 2003; VanderLugt and Lenz, 2008; VanderLugt *et al.*, 2009). All field collections were made at the same site in the southern region of the Bay (e.g. Jungbluth *et al.* (in press), station S3), and all net tows were conducted with a 0.5-m diameter, 64-μm mesh ring net, towed vertically from 10-m depth to the surface. All bulk plankton samples were stored at −20°C after collection.

DNA extraction and qPCR methods

In this study, we measured DNA degradation as a loss in the number of DNA copies individual^{-1} of a specific mitochondrial gene fragment over time, in different preservation solutions. This approach has significant advantages over endpoint PCR for detecting DNA degradation, as it is far more sensitive to small changes in DNA copy number than visualization of band strength on an agarose gel. The DNA extraction and qPCR methods used here are fully described in Jungbluth *et al.* (in press). In brief, sets of 10 adult females were removed from preserved bulk plankton samples and placed directly into 500 μL ATL Buffer for homogenization by bead-beating, followed by DNA extraction using the QiaAmp mini DNA extraction kit (Qiagen). The *DNA extraction from tissues* protocol was applied, with the following modifications: reagent volumes were scaled up for increased starting volume (2.22×), samples were incubated at 56°C overnight for 16–20 h, the recommended RNAse A step was incorporated and DNA was eluted in 400 μL autoclaved deionized water (dH_2O). DNA extracts were then stored at −80°C for between 2 days (laboratory experiment) and 4 months (long-term field experiment) before use in qPCR. The longer storage time for the long-term field experiment was necessary given the design of this experiment, and the need to run all samples on as few qPCR plates as possible (see below). Most samples were thawed only once for measurement of DNA concentration prior to use in qPCR, but the samples run as inter-plate calibrators in the long-term field experiment ranged up to a maximum of four freeze/thaw cycles. Samples used in between-tow comparisons in the short-term field experiment also had four freeze/thaw cycles, due to an initial failed qPCR run (low efficiency).

Our qPCR protocol targets a 165-bp fragment of the mitochondrial gene cytochrome oxidase subunit I (mtCOI), using *P. crassirostris* specific primers PCOI424 (5′-GCG GGA GTA AGA TCA ATT CTA GGC-3′) and PCOI588 (5′-AGT AAT GGC CCC TGC TAA TAC GG-3′) (Jungbluth *et al.*, in press). qPCR reactions were conducted in 50 μL volumes with 25 μL iQ Sybr Green Supermix (Bio-Rad), 0.4 μM of each primer, bovine serum albumin at 0.1 μg μL^{-1}, 11 μL nuclease-free water and 5 μL of DNA extract. A small number of samples from each plate ($N = 4$) were measured for DNA concentration using a Qubit fluorometer (Life Technologies) and high sensitivity assay, and DNA concentration was found to be relatively constant (8–10 ng/mL). Therefore, a set volume of 5 μL of DNA extract was used for all samples, and all unknowns fell within the range of the standard dilution series. Reaction conditions were 95.0°C for 10 min; 50 cycles of 95.0°C for 30 s, 65.0°C for 1 min, 72.0°C for 30 s; followed by 95.0°C for 1 min, with melt curve analysis at the end of each experiment, to verify the presence of a single amplified product. Each qPCR plate was run with a 4-point standard dilution series, using the synthesized primer amplicon as the standard (aliquoted for single use), and with each sample run in triplicate. No-template controls were run in triplicate on each plate. For qPCR data analysis, the threshold level was adjusted manually to 150 RFU to allow comparisons of the sample threshold (Cq) across plates and to ensure that the threshold was consistently set at a low level within the log-linear phase of amplification (Larionov *et al.*, 2005; Bustin *et al.*, 2009). If the standard deviation (SD) of the Cq for triplicate measurements of the same sample was >0.5, the extreme value was considered an outlier and was excluded from subsequent analysis (as recommended by Bio-Rad technical support). To determine DNA concentration in an unknown sample, the threshold cycle (Cq) was plotted with the regression of Cq on $\log_{10}$ pg DNA from the standards of known DNA

concentration. Amplification efficiencies during PCR were >90% in all cases. Inter-run variation in the standard curve across different experiments ranged for slopes between −3.42 and −3.59, and intercepts 13.3 to 15.2, with regression r^2 values >0.99 in all cases. With the exception of the long-term field experiment, all samples to be compared were run on the same qPCR plate, to minimize error from inter-plate comparisons. In the long-term experiment, samples were run on three plates, with four of the same samples run on each plate for inter-run calibrations (coefficient of variation for these samples: 3–9%).

Short-term field study

This study tested for DNA degradation over a 1-month period in samples preserved using (i) bulk preservation in acetone, with no change in preservative following collection, (ii) bulk preservation in acetone, with a change to fresh acetone within 12–24 h of collection, and (iii) a standard ethyl alcohol preservation protocol. In the standard ethyl alcohol preservation, bulk plankton was preserved in the field using 95% non-denatured ethyl alcohol, and the alcohol was changed to fresh within 12–24 h of collection (as in Goetze, 2005, 2010, 2011). Samples for this experiment were collected on three field dates, 1 August, 16 August and 30 August 2012. DNA was extracted from the 30 August samples on 31 August, and the samples collected 1 August and 16 August were extracted on 1 September (as described above), resulting in preservation times of 31 days, 16 days and 1 day following collection and initial preservation.

In this experiment, we also examined sources of variation in mtCOI copy number individual^{-1} across samples. We made replicate measurements of mtCOI copy number individual^{-1} in three sets of animals (N = 10 females) collected in the same plankton tow (same sample jar; within-tow replicates) and from three replicate plankton tows taken on the same date at the same location (between-tow replicates), across all three preservation types. This resulted in a total of nine DNA extracts from each sampling date and preservation type. These replicate samples were used to examine whether mtCOI copy number individual^{-1} is more variable between replicate plankton tows, either due to differences in preservation between jars, or due to biologically real differences in mtCOI copy number individual^{-1} between replicate tows (e.g. patchiness in the plankton in reference to mtCOI copy number individual^{-1}).

Long-term field study

Initial results for the short-term field experiment suggested that there was a trend towards lower DNA copy number over time in all preservation types (as also found in Jungbluth *et al.*, in press), but comparisons in means were non-significant. Therefore, we decided to extend the duration of the study to test for DNA degradation over a 4-month period. The field samples collected on 1 and 30 August 2012 were used in this experiment, including all three preservation types. *Parvocalanus crassirostris* adult females were removed from bulk-preserved plankton samples, and DNA extractions were performed at 1-month intervals (1 October, 2 November and 6 December 2012), in order to obtain profiles of mtCOI copy number individual^{-1} in the same samples over a 4-month storage period.

Laboratory study

Finally, prior work had suggested that food concentration may affect mtCOI copy number in this species (Jungbluth *et al.*, in press), which could potentially mask any effects of DNA degradation in field samples due to natural variation in mtCOI copy number across individuals. In order to control for the influence of a variable food environment on mtCOI copy number, we conducted short-term preservation experiments using animals from laboratory culture that were maintained under constant food concentrations during the experiment. In this experiment, animals in culture were maintained on a monoalgal diet of *Isochrysis galbana* at a constant concentration of 5×10^4 cells mL^{-1} (VanderLugt and Lenz, 2008). Animals were preserved in bulk, using the same three methods outlined above, on 8 October, 23 October and 6 November 2012. Sets of 10 adult females were removed from these bulk samples, and DNA was extracted on 7 November 2012, resulting in storage times of 30, 15 and 1 days following initial preservation.

Statistical design

In the short-term field and laboratory experiments (30-day), we tested for differences in mean mtCOI copy number individual $^{-1}$ for all three preservation types in a two-way ANOVA, followed by multiple comparisons (the Holm–Sidak method). We did not use repeated measures ANOVA in this case because the measurements of mean mtCOI copy number individual^{-1} in samples of different preservation duration were made on different samples (e.g. at day 1, days 15–16, days 30–31). Measurements made at all three time points are shown in Figs 1, 2 and 4. Equal variance of within-tow and between-tow replicate samples in the short-term field experiment was tested using a Levene's test, with inclusion of ethanol and acetone (changed) preserved samples at all three time points (day 1, day 16, day 31). Finally, a

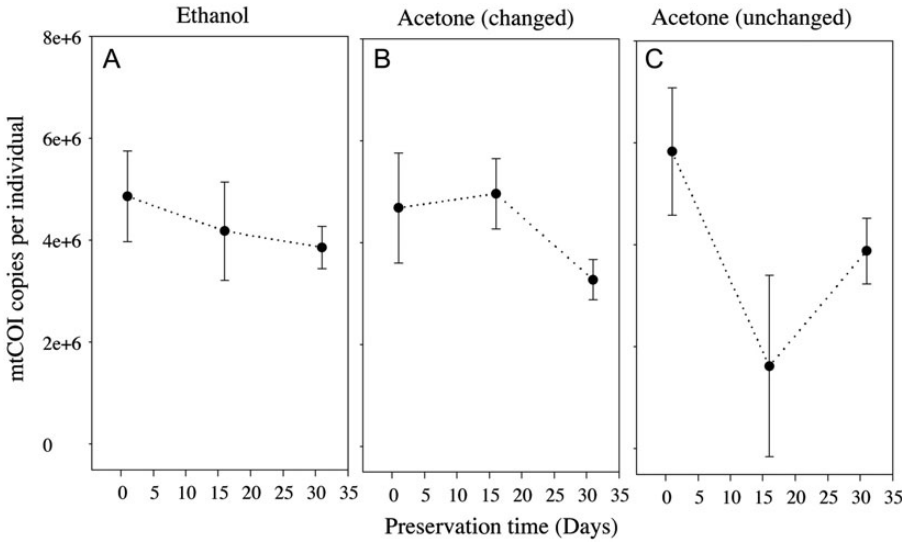

Fig. 1. Short-term field preservation experiment. Changes in DNA copy number individual^{-1} over time of preservation for samples bulk preserved in (**A**) 95% ethanol, with a change to fresh ethanol within 12–24 h of initial fixation, (**B**) acetone, with a change to fresh acetone within 12–24 h of initial fixation, and (**C**) acetone, with no change in fixative. Samples were stored for 1, 16 and 31 days prior to DNA extraction. Error bars are the standard deviation of biological replicates (replicate tows).

repeated-measures ANOVA was used to test for differences in mean mtCOI copy number individual^{-1} across samples of all preservation types in the longitudinal long-term field study.

RESULTS

Short-term field study

DNA preservation over a 30-day storage period was comparable in ethanol-preserved and acetone-preserved samples, when the fixative was changed within 24 h of initial collection (Fig. 1). Both preservation methods had declining DNA copy number individual^{-1} over the 30-day experiment, with starting mean mtCOI copy numbers of 4.9×10^6 and 4.6×10^6, respectively, that dropped to 3.9×10^6 and 3.3×10^6 copies following 30 days in storage at −20°C. In both sample preservation types, this decline in mean copy number was non-significant (two-way ANOVA, multiple comparisons, $P > 0.15$ both cases). However, in acetone (unchanged) preserved samples, the decline was significant (two-way ANOVA, multiple comparisons, $P < 0.02$), suggesting that DNA degradation was occurring during storage in these samples. There was a significant effect of preservation time on mean mtCOI copy number individual^{-1} in this experiment, and also a significant interaction between preservation time and preservation type [ethanol, acetone (changed), acetone (unchanged); two-way ANOVA, $P < 0.05$, both cases]. In summary, the decline in mean copy number individual^{-1} in all sample preservation types suggests that DNA may be degrading during storage in this experiment, but we lack statistical power to conclude this with confidence for the ethanol and acetone (changed) samples.

There was no difference in the level of variation in copy number individual^{-1} observed in the between-tow and within-tow replicate samples in this experiment, in contrast to our expectations. Although the between-tow variance was higher in four of six comparisons (Fig. 2; within versus between, across preservation type and time), these differences were non-significant (Levene's test, $P > 0.2$ in all cases; Fig. 2). There also was no significant difference in variance across preservation types [ethanol, acetone (changed)]. The standard deviations of mtCOI copy number individual^{-1} for ethanol-preserved samples of within-tow and between-tow replicates were $1.0–4.8 \times 10^5$ and $3.9–7.5 \times 10^5$, respectively (for three preservation times; 1, 15, and 30 days). Comparable numbers for acetone-preserved samples were $3.0–9.0 \times 10^5$ and $1.3–8.0 \times 10^5$, for within-tow and between-tow replicates (standard deviations for each time point).

Long-term field study

In the long-term field study, there was a significant effect of preservation time but not preservation type on mtCOI copy number individual^{-1} (two-way repeated-measures ANOVA, $P < 0.002$ and $P > 0.8$, respectively). The overarching result of an effect of preservation time was driven by significant declines in mtCOI copy number

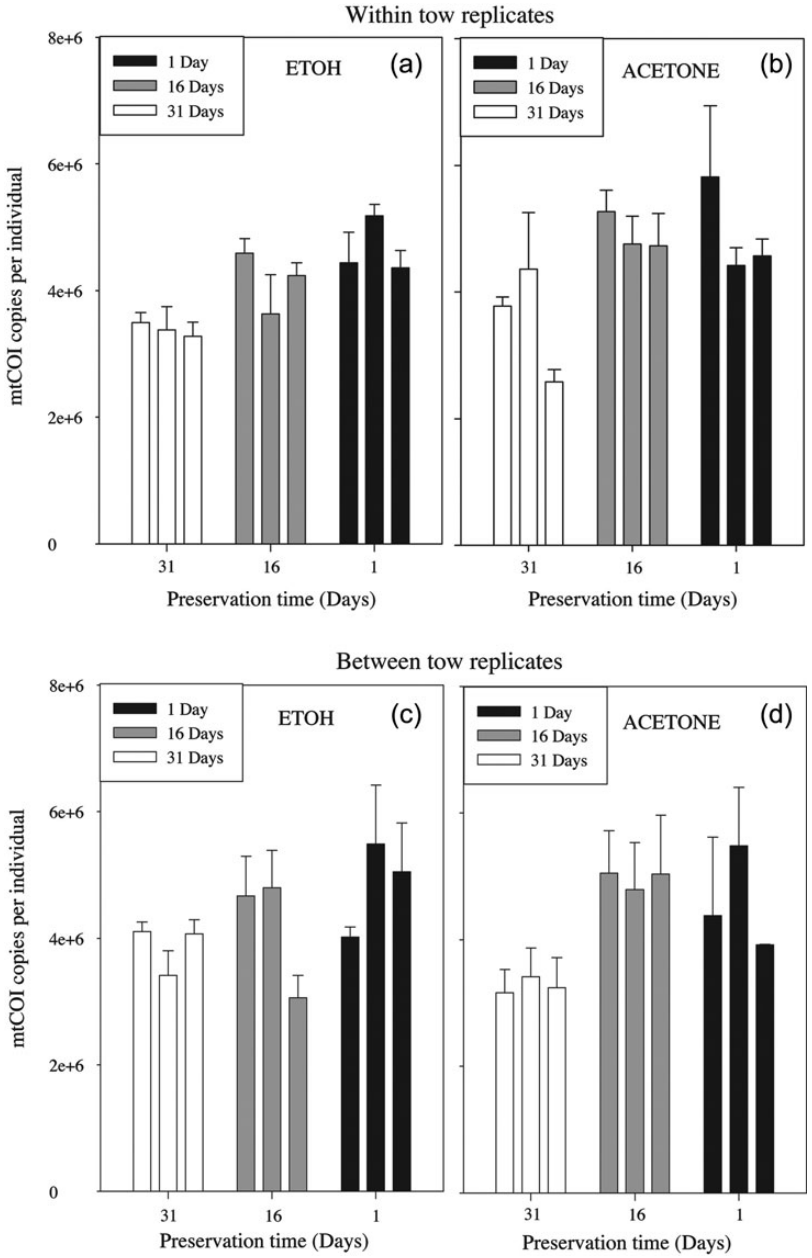

Fig. 2. Comparison of variation in mtCOI copy number individual $^{-1}$ within (**A** and **B**) and between (**C** and **D**) different plankton tows. (**A** and **B**) Each bar represents the mean mtCOI copy number individual^{-1} for $N = 10$ adult females taken from the same plankton tow, and the error bars are the standard deviation of qPCR technical replicates for that sample. (**C** and **D**) Each bar represents the mean mtCOI copy number for $N = 10$ adult females taken from replicate vertical plankton tows taken in succession at the same station in Kāneʻohe Bay, and the error bars are the standard deviation of qPCR technical replicates. In this case, each biological replicate was preserved separately. Animals in A and C were preserved in 95% ethanol, and animals in B and D were preserved in acetone, followed by a change to new acetone within 12–24 h of collection. In all cases, mtCOI copy number was measured after 1, 16 and 31 days in storage.

individual^{-1} over time in the acetone (unchanged) preserved samples (multiple comparisons, $P < 0.02$). Samples preserved for 1 day had significantly higher mtCOI copy number than samples preserved 93 and 127 days, suggesting that DNA degradation was occurring in these samples. However, no evidence was found for DNA degradation over a 4-month period in samples preserved in ethanol and acetone, with the fixative changed within 24 h of collection. Both ethanol and acetone (changed) samples showed declines in mtCOI copy number individual^{-1} over time, as in the short-term field experiment, but the total magnitude of the decline for a 4-month period was

not greater than that in the 1-month study. Ethanol-preserved samples started at a mean of 4.8×10^6 mtCOI copies individual^{-1} after 1 day of preservation and declined to 3.7×10^6 after 127 days in storage. Similarly, in acetone (changed) preserved samples, the mean number of mtCOI copies individual^{-1} was 4.6×10^6 at the start of the experiment and declined to 4.5×10^6 by the end of 4 months. In neither case was the decline significant (two-way repeated-measures ANOVA, multiple comparisons, $P >> 0.05$, both cases). Differences in mean mtCOI copy number individual^{-1} between replicate samples (plankton tows) were comparable to the differences observed between subsequent time points in this experiment (Figs 2 and 3).

Laboratory study

Results for the laboratory preservation study were similar to those for the short-term field experiment, and no evidence was found for DNA degradation occurring over time in this experiment (in this case, in any preservative type). MtCOI copy number individual^{-1} declined over 30 days of storage following preservation in ethanol and acetone (unchanged), dropping from mean copy numbers of 4.27×10^6 and 3.93×10^6, respectively, at the start of the experiment to copy numbers 3.53×10^6 and 3.72×10^6 at the end of the experiment. Acetone (changed) preserved samples actually had higher measured mtCOI copy number at the end of the experiment than at the beginning, a result made possible by the fact that these were measured in distinct samples, preserved 1 month apart in time. Stochasticity in mtCOI copy number between individuals could result in higher DNA content in the samples stored the longest. There was no effect of preservation time or preservation type, nor an interaction of these factors, in this experiment (two-way ANOVA, $P > 0.1$ all sources of variation). The levels of variation seen in the short-term field and laboratory experiments also were broadly comparable (Figs 1 and 4), suggesting that a controlled food environment did not reduce variation in mtCOI number individual^{-1}.

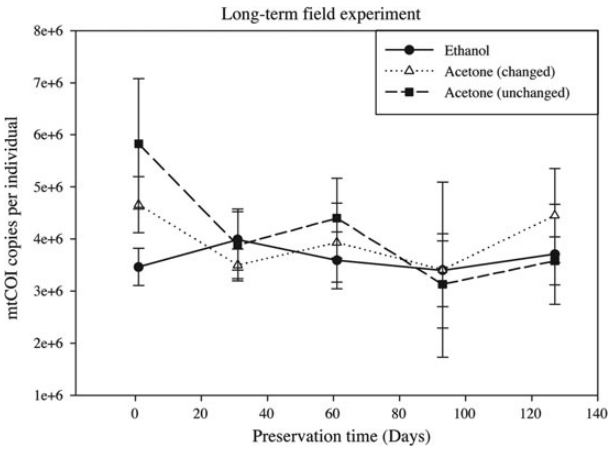

Fig. 3. Long-term field preservation experiment. Changes in DNA copy number individual^{-1} over time of preservation for samples bulk preserved in 95% ethanol, acetone (changed to new acetone within 24 h of collection) and acetone (unchanged). Samples were stored for 1, 31, 61, 93 and 127 days prior to DNA extraction. Error bars are the standard deviation of biological replicates (replicate tows/samples).

DISCUSSION

Preservation in 95–100% ethanol is currently the most commonly used method for bulk fixation of zooplankton samples for molecular studies. However, ethanol is known to be an imperfect fixative (e.g. Reiss *et al.*, 1995; Fukatsu, 1999), and alternative solutions are desired that would yield better preservation of DNA over medium to long storage times (months to years). As outlined in the introduction, acetone is in widespread use as a fixative for terrestrial arthropods, and is particularly promising for use in biological samples that have high water content. In this study, we demonstrated that acetone provides comparable, but not superior, DNA preservation in planktonic copepods over periods of weeks to months, following bulk fixation of plankton samples and replacement of the fixative within 24 h of collection. Modest declines in mtCOI copy number individual^{-1} were observed in both acetone (changed fixative) and ethanol-preserved samples, but the magnitude of the decline was approximately the same in samples preserved and stored for 1 month and 4 months, suggesting that initial declines in mtCOI copy number do not continue over time during sample storage. These modest declines may be important in some quantitative and semi-quantitative applications (e.g. Durbin *et al.*, 2008; Nejstgaard *et al.*, 2008; Simonelli *et al.*, 2009; Cleary *et al.*, 2012), and should be considered in the design of field programs that include bulk fixation of samples. However, although DNA preservation in acetone (changed fixative) was comparable to that in ethanol, a precipitate formed in the presence of seawater in the acetone-preserved samples that covered many specimens, making them difficult to identify to species. Copepods preserved in acetone also were more brittle than those preserved in ethanol, and animals were easily damaged during handling (e.g. broken limbs). Finally, acetone is more toxic to humans, decreasing the ease of use during bulk fixation. As a result, we do not recommend acetone as a bulk fixative for zooplankton samples. However, acetone may be very effective for DNA preservation of single specimens for some taxonomic groups, and should be explored as an alternative for taxa that are known to preserve poorly in ethanol (e.g. planktonic

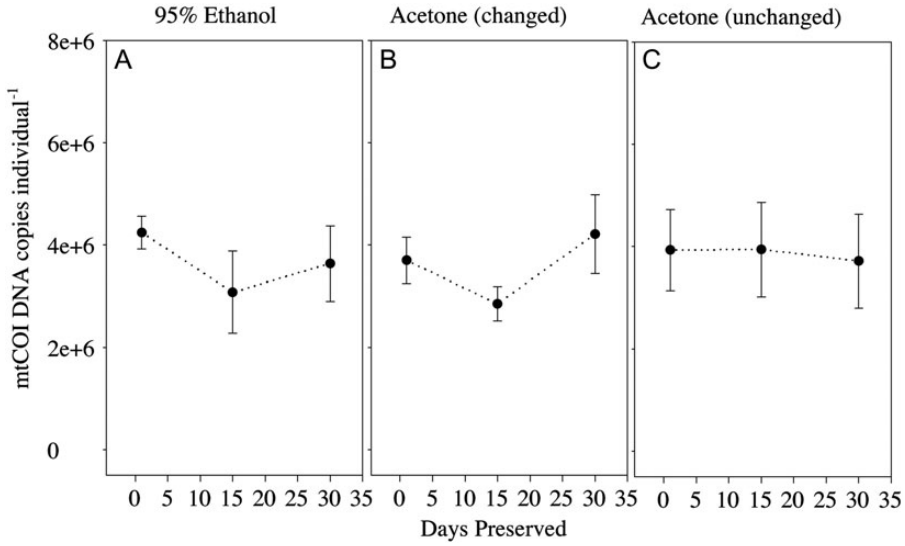

Fig. 4. Short-term laboratory experiment, with food levels maintained at 5×10^4 cells ml^{-1} over the 30-day experiment. Changes in DNA copy number $individual^{-1}$ over time of preservation for culture samples bulk preserved in (**A**) 95% ethanol, with a change to fresh ethanol within 12–24 h of initial fixation, (**B**) acetone, with a change to fresh acetone within 12–24 h of initial fixation, and (**C**) acetone, with no change in fixative. Samples were stored for 1, 15 and 30 days prior to DNA extraction. Note that the *y*-axis scale in these plots is the same as in Fig. 1.

chaetognaths, cnidarians; Dawson *et al.*, 1998; Peijnenburg *et al.*, 2004; Peijnenburg *et al.*, 2006). Changing the acetone fixative was found to be an important step to ensure the integrity of DNA, as significant DNA degradation was observed in the long-term (4 months) experiment in acetone-preserved samples that did not have the fixative changed.

This study also provided the first qPCR-based assessment of DNA degradation in ethanol-preserved samples for marine invertebrates, and our results demonstrate that degradation is modest over short (weeks) to medium-term (months) time periods in bulk-fixed samples that were stored at −20°C. These results were unexpected, given both our preliminary observations of declines in mtCOI copy number $individual^{-1}$ in the short-term field experiments (this study, Jungbluth *et al.*, in press), and also given our prior experience with low success rates for microsatellite marker PCR amplification in older ethanol-preserved samples (>5 years, Andrews and Goetze, unpublished data). We expected to observe a clear and consistent decline in mtCOI copy number $individual^{-1}$ over time in storage. Given the results of this study, the ease of sorting and identifying specimens from ethanol-preserved material, and the relatively low cost of this preservative, ethanol remains one of the best chemical preservatives for field studies that require immediate bulk fixation of zooplankton samples. However, DNA preservation in ethanol is sensitive to dilution by seawater (Fukatsu, 1999), and poor preservation in ethanol often occurs when samples have high plankton to ethanol volumes or the ethanol is not changed promptly within 24 h of collection (Goetze, unpublished observation). Successful use of ethanol preservation in larger field surveys therefore requires that care be taken to prevent ethanol dilution during bulk fixation.

Finally, many molecular studies still require better preservation of nucleic acids than can be obtained using bulk fixation in either acetone or ethanol. Cryopreservation and storage in RNALater® are still the methods of choice for work with RNA and any work that is unforgiving in its requirement for HMW DNA (e.g. next generation sequencing; Bucklin, 2000; Gorokhova, 2005). However, despite the superiority of these preservation methods, they remain intractable in many cases, because they require immediate sorting and identification of specimens in the field. Community-wide molecular studies from large-scale field programs cannot be conducted using live sorting methods, and ethanol still appears to be the best bulk fixative in such cases.

ACKNOWLEDGEMENTS

We thank P. Lenz for assistance with and access to the *Parvocalanus crassirostris* cultures maintained in her laboratory.

FUNDING

This work was funded in part by National Science Foundation grant OCE-1255697 (to E.G.), and in part by a grant/cooperative agreement from the National Oceanic and Atmospheric Administration, Project R/

HE-18, which is sponsored by the University of Hawaii Sea Grant College Program, SOEST, under Institutional Grant No. NA09OAR4170060 from NOAA Office of Sea Grant, Department of Commerce. The views expressed herein are those of the author(s) and do not necessarily reflect the views of NOAA or any of its subagencies. UNIHI-SEAGRANT-JC-12-27.

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