The Effect of Silica Desiccation Under Different Storage Conditions on Filter-Immobilized Environmental DNA

Michael J Allison  
University of Victoria

Jessica M Round  
University of Victoria

Lauren C Bergman  
University of Victoria

Ali Mirabzadeh  
Bureau Veritas Canada Inc

Heather Allen  
Bureau Veritas Canada Inc

Aron Weir  
Bureau Veritas Canada Inc

Caren Helbing (chelbing@uvic.ca)  
University of Victoria https://orcid.org/0000-0002-8861-1070

Research note

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Abstract

Objective

Silica gel beads have promise as a non-toxic, cost-effective, portable method for storing environmental DNA (eDNA) immobilized on filter membranes. Consequently, many ecological surveys are turning to silica bead filter desiccation rather than ethanol preservation. However, no systematic evaluation of silica bead storage conditions or duration past one week has been published. The present study evaluates the quality of filter-immobilized eDNA desiccated with silica gel under different storage conditions for over a year using targeted quantitative real-time polymerase chain reaction (qPCR)-based assays.

Results

While the detection of relatively abundant eDNA target was stable over 15 months from either ethanol- or silica gel-preserved filters at -20 and 4°C, silica gel out-performed ethanol preservation at 23°C by preventing a progressive decrease in eDNA sample quality. Silica gel filter desiccation preserved low abundance eDNA equally well up to 1 month regardless of storage temperature (18, 4, or -20°C). However only storage at -20°C prevented a noticeable decrease in detectability at 5 and 12 months. The results indicate that brief storage of eDNA filters with silica gel beads up to one month can be successfully accomplished at a range of temperatures. However, longer-term storage should be at -20°C to maximize sample integrity.

Introduction

Over the past decade, techniques used to detect environmental DNA (eDNA) – genetic material present in environmental samples from secretions, excretions, exogenous sloughing of eukaryotic cells, or from microscopic organisms (1) – have surged in use by academic, government, conservation, and development sectors for providing cost-effective information about at-risk and invasive species in natural and managed ecosystems (2–5). In eDNA-based surveys, a common technique is to immobilize eDNA from water samples on filter membranes, extract the DNA, and perform targeted taxa quantitative real-time polymerase chain reaction (qPCR) analyses. Ideally, these filters can be stored for some time or archived prior to DNA analysis. Filters are typically immersed in high percentage, molecular grade ethanol to prevent sample degradation during storage (6). Immersion of filters in ethanol at room temperature for up to two weeks gave better eDNA performance than directly freezing filters with no preservation or extracting DNA from filters within 5 h of filtration at room temperature (7). While relatively straightforward to use, the use of ethanol presents several challenges in that it is a dangerous good (volatile, flammable, and poisonous) requiring special shipping permits, and adds considerable bulk and weight to the field sample. However, recently, the use of silica gel beads as a filter desiccant have been suggested as a lightweight alternative to ethanol immersion (8–10). Despite this suggestion, little empirical work has
been published evaluating silica gel filter storage conditions under different temperatures over periods greater than one week.

The present study evaluates the quality of filter-immobilized eDNA desiccated with silica gel beads for over a year under different storage temperatures. In the first experiment, targeted detection of relatively abundant (~50,000 copies per reaction) eDNA from an outdoor fresh water tank was examined using filters preserved in ethanol or silica gel beads for up to 15 months at 23, 4, and −20°C. In the second experiment, low abundance (~500 copies per reaction) eDNA targets from water samples spiked with a dilute tissue slurry were tracked using two separate targeted qPCR assays on filters preserved by silica gel beads for up to 12 months at 18, 4, and −20°C.

Methods

Animal care and handling

A single premetamorphic American bullfrog (*Rana [Lithobates] catesbeiana*) tadpole was used as a source of bullfrog tissue to create a standard slurry detailed below. The tadpole was euthanized using 0.1% (w/v) tricaine methane sulfonate (Syndel Laboratories, Nanaimo, BC, Canada) buffered in 25 nM sodium bicarbonate (Sigma Aldrich, Canada).

Filter storage in silica gel beads

All procedures were performed in an amplicon-free area and the benchtop was wiped with 10% bleach (v/v) (Javex 12 by Clorox) solution followed by 70% ethanol (v/v) prior to setup. Personnel wore personal protective equipment including nitrile gloves, safety glasses, and a lab coat. Forceps were submerged in 50% bleach (v/v) and thoroughly rinsed with distilled water and dried between handling each sample. Sample water (details are provided for the individual experiments below) was vacuum filtered through Nalgene analytical test filter funnels with 0.45 µm mixed cellulose ester filters (Thermofisher Scientific Inc., Mississauga, ON, Cat#145–2045). Mixed cellulose ester (cellulose nitrate and acetate mixture) was chosen because it, along with cellulose nitrate, empirically gave the highest DNA yield when comparing filter membrane compositions (7, 9, 11, 12). The vacuum was left on each filter for one minute after the sample had passed through in order to remove excess water. Filters were preserved whole to replicate the effects of disturbance during repeat DNA isolations.

Using forceps, the filter was folded in half with the filtride facing inward and inserted into a pre-labelled Manila paper coin envelope (Fig. 1). This was then inserted into a small sealable plastic bag to which 15–30 mL of color-indicating 2–4 mm rechargeable silica gel beads (Dry & Dry, Amazon, Canada; Product #CRH-16036) were added. The orange beads will turn dark green when they are 50–60% water saturated allowing for easy monitoring of desiccation conditions during storage. The coin envelope prevents direct contact between the filter and the silica beads and protects the filter from damage. The filters, thus prepared, were stored in the dark at the indicated temperatures.
Experiment 1: Comparison between ethanol and silica filter preservation of relatively abundant eDNA at three holding temperatures over time

One-litre samples (n = 2–3 per temperature group) of recirculated water were obtained on the same day at the University of Victoria's Aquatics Facility from an outdoor fiberglass tank used for holding bullfrog tadpoles fed daily with Spirulina. Water was filtered and the filters handled as described above for silica gel desiccation or folded in quarters and placed into a 2 mL microcentrifuge tube containing 95% ethanol (8). The tube was completely filled with ethanol and wrapped in Parafilm for storage to prevent evaporation. One set of filters were not preserved and, rather set aside for immediate processing. DNA was extracted from the filters immediately or after 1, 4, 8, or 15 months of storage at -20, 4, and 23 °C. At each time-point, each filter was individually removed from its coin envelope in a laminar flow hood, and a quarter partitioned off at room temperature. The remaining filter was returned to its coin envelope or the tube containing ethanol and placed back into its designated storage condition.

Experiment 2: Effect of holding temperature and time on low abundance eDNA on silica-preserved filters

A standard eDNA slurry was prepared to ensure that the target DNA was present but diluted to an abundance closer to typical eDNA samples. The slurry was created by mixing a 4 mm diameter dorsal tail fin punch taken from an American bullfrog (*Rana (Lithobates) catesbeiana*) tadpole, a 4 mm diameter flake of Spirulina with 1 mL DNase-free TE buffer pH 8. The mixture was homogenized for 6 min at 24 Hz in a Retsch MM301 mixer mill (ThermoFisher Scientific Inc.) in microtubes containing a 3 mm tungsten carbide bead (the mixer mill rack was rotated 180° halfway through homogenization) to create a standard slurry ([Supplementary Fig. 1](#)).

Since the purpose of this experiment was to monitor DNA integrity on filters over time measured by C_t values, it was crucial for all initial time points to have a positive detection. In order to determine the lowest concentration of DNA that resulted in 100% positive detection in all of the technical replicates, a 10-fold serial dilution test using recirculated fresh water from the Aquatics Facility was carried out and the appropriate slurry dilution (10^{-6}) was selected to create a 2L 10X working stock ([Supplementary Fig. 1](#)). Three hundred milliliters of this working stock was added to each of five replicates of 2,700 mL recirculated water and each of these 3 L replicates was further divided into 1 L aliquots that were individually vacuum filtered ([Supplementary Fig. 1](#)). The end result was three filters with identical filtride which were distributed between each storage temperature (18, 4, and –20°C; [Supplementary Fig. 1](#)). Each 1 L experimental sample was matched by a 1 L negative control sample of bottled distilled water (Equate brand, Walmart) for a total sample number of 30. In a laminar flow hood, all filters were quartered using forceps that had been bleach-treated and rinsed with copious amounts of distilled water before and after handling each sample. Each quarter filter was stored in a separate coin envelope in order to allow for consistent preservation conditions and preserved using silica gel beads as described above.
DNA isolation and analysis

The DNA from one quarter of each filter was isolated using the DNeasy Blood and Tissue kit (QIAGEN Inc., Mississauga, ON, Canada; Cat# 69506) following the procedure outlined in (13). The test for relatively abundant chloroplast signal using the IntegriT-E-DNA™ assay as described previously (13, 14) and the tests for low abundance bullfrog DNA (eFrog3 and eLICA1) were previously validated and described (13). An additional validation step for these two assays was performed using gBlocks® synthetic DNA ordered from Integrated DNA Technologies (Coralville, Iowa, United States) following the method outlined previously (14). This allowed creation of a standard curve relating qPCR cycle threshold values to starting copy numbers, and objective, standardizable comparison of assay results. All qPCR tests followed the same run conditions outlined in (13) except that the eFrog3 assay 30 second annealing step was adjusted to 60 °C. All distilled water filter controls, positive plate controls, and no template plate negative controls never produced a signal.

Statistical analyses

The qPCR data was analyzed with R Studio© version 1.2.1335 (2009–2019 R Studio, Inc). Data are expressed as median values to reduce the influence of outlier measurements and plotted with median absolute deviation error. Median C_t values for each set of eight technical qPCR replicates representing the DNA from one quarter of a filter were transformed to copies per reaction using the formula derived from each assay’s synthetic DNA standard curve (Supplementary Fig. 2). The median copy-per-reaction values were assessed for normality using the Shapiro-Wilk test and homogeneity of variance using Levene’s test. After determining that requirements for normality and homogeneity were not met, non-parametric analyses were carried out. The Friedman repeated measures test was used to determine whether test groups contained significant differences (p ≤ 0.05), and the Wilcoxon Signed Rank test was used to determine pairwise significance between treatments within each group (p ≤ 0.06).

Results And Discussion

Comparison of ethanol and silica gel preservation methods with relatively abundant eDNA

In the first experiment, we evaluated eDNA samples for relatively abundant, naturally-occurring chloroplast DNA found in water samples obtained from an outdoor tank. As expected, the filters that were immediately processed (“None” Fig. 2) returned C_t values between 20–23 (corresponding to ~ 50,000 copies/reaction) for intact samples consistent with previous observations with field water samples (14, 15). Filters that were stored in ethanol at 23°C experienced a progressive decrease in eDNA sample quality (as demonstrated by the shift in C_t value) across all time points compared to filter samples that were processed immediately. This sample degradation was not seen in the filters that were stored with silica gel beads (Fig. 2). The detection rates of DNA isolated from the 23°C silica gel bead preserved filters and the filters stored at -20 or 4°C in either ethanol or silica gel beads at any time point up to 15
months were stable as indicated by the consistent Cₜ values (Fig. 2). Majaneva et al. (2018) compared ethanol to silica gel bead preservation methods for filters destined for metabarcoding analyses. They only analyzed one storage condition and time point (room temperature for one week) and found that silica gel desiccation yielded more consistent community composition than ethanol despite the ethanol samples having a higher concentration of DNA extracted from the filter (9). Our results indicated that for relatively abundant target DNA, ethanol and silica beads performed comparably over the short-term at cooler temperatures, but sample quality deteriorated in ethanol at 23°C when stored greater than one month. At this temperature, we also observed that some ethanol-preserved filters began to physically degrade after one month of storage, and the ethanol in several samples evaporated, severely compromising the sample DNA.

**Effect of holding temperature on low abundance eDNA detection on silica-preserved filters over time**

Across the three different temperature conditions, the eFrog3 test detected significant loss in DNA copy numbers over 12 months at 18 °C and the eLICA1 measured significant loss at both 18 °C and 4 °C (Friedman; p = 0.05; Fig. 3). Low abundance eDNA was preserved equally well up to 1 month regardless of storage temperature. However, a noticeable decrease in detectability was observed at 5 and 12 months when filters were stored at 18 and 4°C (Wilcoxon; p = 0.06). Sample integrity was maintained to 12 months when filters were stored at -20°C (Fig. 3).

**Conclusions**

Silica gel beads are an excellent alternative to ethanol for preservation of eDNA filter samples. While short-term storage in the dark up to one month can be performed at warmer temperatures, longer-term storage should be at -20°C to maintain sample quality prior to DNA isolation.

**Limitations**

We used three separate targeted qPCR assays and evaluated relatively abundant and low abundance DNA targets to assess the effectiveness of silica gel bead desiccation of eDNA immobilized on filters for eDNA analysis. The assessment of additional targets from a wider variety of water samples is desired.

**Abbreviations**

- **eDNA**: environmental deoxyribonucleic acid
- **EtOH**: ethanol
- **qPCR**: quantitative real time polymerase chain reaction

**Declarations**
Ethical approval and consent to participate

All animal procedures were carried out under the guidelines of the Canadian Council on Animal Care and guidance of the Animal Care Committee, University of Victoria under an approved protocol. No human studies were performed.

Consent to publish

All authors consent to the publication of this work.

Availability of data and materials

All data generated and analyzed in the present study are included in the published article and supplementary information.

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

All authors designed the research; MJA, JMR, LCB, AM, HA performed the experiments; MJA, JMR, LCB, CCH analyzed the data; MJA, LCB, and CCH prepared the manuscript. All authors read and approved the final manuscript.

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