Comparison of the effect of time and preservative on the quality of DNA from pitfall traps

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Further information

This report can be downloaded from the Natural England Access to Evidence Catalogue: http://publications.naturalengland.org.uk/. For information on Natural England publications contact the Natural England Enquiry Service on 0300 060 3900 or e-mail enquiries@naturalengland.org.uk.

Foreword

DNA – based methods offer a significant opportunity to change how we monitor and assess biodiversity. However, for most techniques, there is still development required before they can be used in routine monitoring. Natural England has been exploring the further use of these methods for environmental monitoring for several years, delivering a series of reports which focus on the development of DNA-based methods with potential in a particular area.

A recent literature review (NECR423) has investigated the current state of knowledge for sampling and preservation of terrestrial invertebrates for analysis via DNA techniques. Here we continue this work in a field- and lab-based study comparing several different methods of invertebrate trapping and preservation and assessing the quality and quantity of DNA yielded. This work aims to develop standardised best practise methods for the trapping of terrestrial invertebrates for subsequent DNA analysis by Natural England staff.

Natural England commissions a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Executive summary

The aim of this project was to generate evidence to inform best practise guidance for Natural England staff wishing to use DNA metabarcoding to identify invertebrate communities from pitfall trap samples. Specifically, we wished to determine whether ethanol or propylene glycol should be used as the preservative solution for optimum preservation of invertebrate DNA, considering results and practicalities in the field. We also set out to determine what length of time traps could be left out in the field and still retain high quality invertebrate DNA; and whether the use of 'roofs' over pitfall traps would affect the DNA quality. To do this, we designed an experiment in which 48 pitfall traps were set up containing either ethanol, propylene glycol or water, some with roofs and some without, and left these in the field for differing lengths of time from 0 – 21 days. Bulk invertebrate samples from these traps were then sent to the laboratory for DNA extraction, analysis of DNA yield and purity, and PCR with two sets of invertebrate specific primers. These analyses showed that trap samples collected in ethanol yielded less DNA the longer they were left in the field, and this DNA also became more degraded at the longer timepoints. Trap samples collected in propylene glycol however yielded relatively consistent amounts of DNA, which did not become significantly more degraded over time (up to 21 days). As expected, samples collected in water gave very low yields of highly degraded DNA. In this study, traps with roofs gave higher yields of DNA particularly when using propylene glycol, but roofs were not shown to improve DNA fragmentation or PCR. However, the applicability of roofs will depend on weather conditions and invertebrate taxa required. All samples collected in ethanol and propylene glycol in this study showed good amplification with invertebrate specific primers, with minimal differences in Cq values, demonstrating their suitability for metabarcoding applications. Considering the higher DNA yields; lower degradation; and ease of use in the field (as compared to ethanol), we would recommend the use of propylene glycol for preservation of pitfall trap samples for DNA analysis. DNA metabarcoding has excellent potential for invertebrate identification and monitoring. This study has provided evidence to inform best practise guidance for the setup of pitfall traps to be analysed using DNA metabarcoding techniques.

Contents

1. Introduction

Natural England is the Government's adviser for the natural environment. It provides practical advice on how to safeguard England's natural wealth for the benefit of everyone. ADAS is an environmental consultancy which exists to provide ideas, specialist knowledge and solutions to secure our food and enhance the environment.

Natural England wishes to recommend best practice to staff undertaking invertebrate surveying using pitfall traps and DNA sequencing in the future. Proof of concept studies performed previously (NECR388) have demonstrated that DNA could change the way that some terrestrial invertebrate monitoring is carried out. However, a recent review of peer reviewed, and grey literature (NECR423) has demonstrated that there was little evidence to support recommendations of the best preservative solution to use in traps to maximise DNA quality. Natural England has therefore run a small study where pitfall traps were left out for different lengths of time with different preservatives and with or without roofs; prior to investigating the quality of the DNA returned in order to recommend best practice to Natural England staff carrying out future terrestrial invertebrate studies that will utilise DNA methodologies such as community analysis via metabarcoding.

1.1 Aims and objectives

The overall aim of this study is to determine the effect of storage conditions on the DNA contents of pitfall traps collected for different lengths of time and with different preservative solutions. Investigation of any measurable change in the quantity and quality of the DNA recovered from the samples and whether any change can be associated with sampling and preservative methodology was carried out. qPCRs were carried out using invertebrate primers to monitor the cycle thresholds at which PCR amplification commences as a measure of the success of each PCR.

This report details the methodology employed in this study, the results obtained, and discussion of the results followed by recommendations. Results from this study will inform best practice guidelines for invertebrate collection using pitfall traps to be applied by Natural England in future surveys.

2. Materials and methods

2.1 Sample collection

Natural England survey protocols were used to collect pitfall trap samples at Draycott, Cheddar (ST4854451319), a limestone grassland. A total of 48 pitfall traps were set up, based on Sadler and Bell (2000). 250mL plastic cups were dug into the ground such that their rims were flush with the surface. Traps were part filled with 80mL of either 95% ethanol; 99% propylene glycol; or tap water (see table below). Some traps additionally had

a roof, made by securing a bamboo plate above the trap using nails. Pitfall traps were set up in a staggered timeframe and left in the field for between 21 and 3 days (see Table 1 for trap set up). All pitfall trap samples were then collected and processed on the same day, at which time control samples (0 days) were also collected. Upon collection, specimens from each trap were transferred into fresh preservative (ethanol or propylene glycol, respectively) in either a 15 mL or 50 mL tube such that one trap equalled one sample contained in one tube. All samples were collected from Natural England on 19th May 2022 and transported to the ADAS laboratories for processing. Sample processing took place between 27/05/2022 and 13/06/2022, therefore the average number of days between collection and DNA extraction was 12 days.

Number of days (time)	Pitfalls set up without roof		Pitfalls set up with roof		Water Controls
	Ethanol	Propylene Glycol	Ethanol	Propylene Glycol	
21	E1, E2, E3	PG1, PG2, PG3	N/A	N/A	N/A
15	E4, E5, E6	PG4, PG5, PG6	ER4, ER5, ER6	PGR4, PGR5, PGR6	W1, W2, W3
10	E7, E8, E9	PG7, PG8, PG9	N/A	N/A	N/A
8	E10, E11, E12	PG10, PG11, PG12	ER10, ER11, ER12	PGR10, PGR11, PGR12	W4, W5, W6
3	E13, E14, E15	PG13, PG14, PG15	N/A	N/A	N/A
Handpicked Controls (0)	EC1, EC2, EC3	PGC1, PGC2, PGC3	N/A	N/A	N/A

Table 1. Pitfall trap setup record.

2.2 Laboratory standard and specification

All laboratory activities associated with DNA analysis are subject to errors if quality control is inadequate. Our DNA analysis follows a unidirectional workflow with separate laboratories and staff to act as a physical separation for the different aspects of the analysis work. This greatly reduces the potential for contamination of samples or the PCR amplicons. ‘Blank’ PCRs (sterile water rather than DNA) are used to monitor for reagent/procedural contamination, and in addition positive control samples are used to increase confidence in the results and identify any cross-contamination issues, should they occur.

2.3 DNA extraction and quantification

Each sample (i.e. the contents of one pitfall trap) was individually transferred to fresh sterile petri dishes to air dry within a laminar flow hood prior to processing. Samples provided in propylene glycol were first rinsed in ethanol to remove residual propylene glycol prior to drying. Where large specimens were present (large slugs, snails and beetles) a small piece was cut from the specimen for use in the DNA extraction. Each sample was then individually transferred to a clean, sterile mortar and ground into a powder using a pestle and liquid nitrogen. Ground up samples were transferred into 7 mL bijoux tubes before 3x 25 mg was weighed and transferred into 1.5 mL Eppendorf tubes for subsequent DNA extraction. Bijoux tubes containing the remaining sample were then stored at -20°C.

DNA was extracted in triplicate on a set weight of dry matter (25 mg) using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (described below) except that an overnight incubation at 56°C was carried out to ensure complete proteinase K digestion of the samples. Final resuspension was in 200 µL AE buffer. All extractions were quantified, and purity measurements made using a Qubit 3.0 Fluorometer (Invitrogen) or NanoDrop Spectrophotometer (Thermo Fisher) (respectively) following the manufacturer's instructions. All remaining DNA was stored at -20 ºC prior to PCR set up (described below).

DNA extraction:

1. Add 180 µL of pre-warmed ATL buffer and 20 µL PK from the DNeasy Blood and Tissue kit to each 1.5 mL Eppendorf tube and vortex to ensure samples are properly mixed.
2. Place all sample in a water bath at 56°C overnight to ensure that all samples are fully digested.
3. After incubation, spin samples for 30 seconds at 6000xg to remove any condensation from the lid.
4. Add 200 µL of AL buffer to each sample and vortex to mix, then incubate in a water bath at 56°C for 10 minutes.
5. Add 200 µL of molecular biology grade ethanol to each sample and mix by vortexing.
6. Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube.
7. Centrifuge at ≥ 6000 xg (8000 rpm) for 1 min. Discard the flow-through and collection tube.
8. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1.
9. Centrifuge for 1 min at ≥6000 xg. Discard the flow-through and collection tube.
10. Place the spin column in a new 2 mL collection tube, add 500 µL Buffer AW2.
11. Centrifuge for 3 min at 20,000 xg (14,000 rpm). Discard the flow-through and collection tube.
12. Transfer the spin column to a new pre-labelled 1.5 mL microcentrifuge tube.
13. Elute the DNA by adding 200 µL Buffer AE to the centre of the spin column membrane. Incubate for 1 min at room temperature (15–25°C).
14. Centrifuge for 1 min at ≥6000 xg.

DNA quantification:

DNA extracts were quantified using the Qubit® dsDNA BR assay kit and Qubit 3.0 fluorimeter as follows:

1. The Qubit® working solution was prepared by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer.
2. Make up two standards by adding 190 µL Qubit® working solution into each of two tubes before adding 10 µL of each Qubit® standard to the appropriate tube. Mix by vortexing.
3. For each extract make up a tube with a final volume of 200 µL containing 2 µL extract and 198 µL Qubit® working solution.
4. Allow all tubes to incubate for two minutes before reading the standards and extracts on the Qubit® 3.0 fluorimeter.

DNA purity:

DNA extracts were measured for their purity using an A_{260}/A_{280} measurement on a Nanodrop spectrophotometer. 2 µL of DNA from each sample was analysed following the manufacturer's instructions. The nanodrop instrument was first calibrated with a blank sample containing the elution buffer only.

2.4 DNA size fractionation

Where possible 150 ng genomic DNA extracted from the samples was run on a 1.5% agarose gel containing Nancy520 at the manufacturers stated concentration at 80mA for 2 hours. A few samples had low DNA concentrations when quantified so it was not possible to run 150ng DNA in these cases: for E2, W2, and W5 50ng was loaded onto the gel; for W4 60ng; for W4 and W6 80ng; and for E5 and E6 100ng. Gels were visualised using a SynGene GelDoc system (SynGene).

2.5 PCR amplification

In order to demonstrate the applicability of samples for both barcoding and metabarcoding analyses, two primer sets were used. Shorter amplicon generation for 'metabarcoding' analysis was carried out using the primer combination FwhF2/FwhR2 (Table 2) and longer amplicon generation for 'barcoding' analysis was carried out using the Folmer primers (Table 2).

Primer	Sequence (5'-3')	Amplicon Size	Reference
FwhF2	**TCGTCGGCAGCGTCAGATGTGTATAAG AGACAG**GGDACWGGWTGAACWGTWTA YCCHCC	205bp	Vamos (2017)
FwhR2	**GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG**GTRATWGCHCCDGCAARWAC WGG	205bp	Vamos (2017)

LCO1490	GGTCAACAAATCATAAAGATATTGG	680bp	Folmer (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	680bp	Folmer (1994)

Table 2. Primers used for PCR amplification. FwhF2/FwhR2 contained the Illumina adapter sequences (**in bold**) to mimic PCR amplification for metabarcoding.

Shorter amplicon generation:

PCRs were set up in duplicate in a total volume of 25 µl consisting of:

a. 3 µL of extracted template DNA (1 ng/µl),
b. 2.5 µL of each primer (0.025 µmol/L),
c. 12.5 µL of iTaq Sybr Green (Bio-Rad),
d. 4.5 µL ddH2O.

PCR Touchdown cycling:

Initial incubation for 5 minutes at 95°C

a. denaturation at 95°C for 30 seconds
b. annealing at 68°C for 1 minute 30 seconds
c. extension at 72°C for 30 seconds

a – c: 15 cycles, reducing by 1°C each cycle to 54°C

Extension 72°C for 2 minutes

d. denaturation at 95°C for 30 seconds
e. annealing at 54°C for 1 minute 30 seconds
f. extension at 72°C for 30 seconds

d – e: 30 cycles

Melt curve step from 65-95°C

Holding at 4°C until collection of PCR products for analysis.

Longer amplicon generation:

PCRs were set up in duplicate in a total volume of 25 µl consisting of:

e. 5 µL of extracted template DNA (1ng/µl),
f. 1 µL of each primer (0.1 µmol/L),
g. 12.5 µL of iTaq Sybr Green (Bio-Rad),
h. 5.5 µL ddH2O.

PCR cycling was as follows:

Initial incubation for 5 minutes at 95°C

a. denaturation at 95°C for 1 minute
b. annealing at 40°C for 1 minute
c. extension at 72°C for 1 minute 30 seconds

a – c: 35 cycles

Extension at 72°C for 1 minute 30 seconds

Melt curve step from 65-95°C

Holding at 4°C until collection of PCR products for analysis.

3. Results and discussion

3.1 DNA recovery

DNA was successfully recovered (triplicate extractions) from each of the pitfall trap samples provided (Table 3). Images of the samples can be found in Appendix 1. All the samples were quantified by Qubit and DNA yield (ng/ul) for each sample is shown in Table 3. The mean yield for each sample storage solution and for each timepoint were recorded and plotted in Figure 1. The amounts of DNA recovered ranged from 1.83ng/ul to 160ng/ul (total of 366ng to 32µg per extraction), mean of 41ng/µl across all extractions. The results showed that amounts of recovered DNA generally decreased as length of sampling increased and overall, there tended to be a greater yield of DNA from samples stored in propylene glycol than ethanol at the time periods greater than 10 days (Figure 1). DNA recovery at time periods of less than 10 days was broadly similar for both ethanol and propylene glycol. There were significant differences in the yield of DNA as determined by one-way ANOVA: ($F(1,16) = 82.9097$, $p = 0$) for 10 days samples; ($F(1,16) = 26.189$, $p = .0001$) for 15 day samples; and ($F(1,16) = 51.1469$, $p = 0$) for 21 day samples. DNA recovery at time periods of less than 10 days was broadly similar for both ethanol and propylene glycol with no significant differences as determined by one-way ANOVA ($F(1,16) = 1.2384$, $p = .2822$), ($F(1,16) = 0.3564$, $p = .5607$) for samples collected for 8 days or 0 days respectively; and a small but significant difference as determined by one-way ANOVA ($F(1,16) = 4.7532$, $p = .0445$) for samples collected for 3 days.

For the samples collected from pitfall traps with roofs there was a greater yield of DNA recovered compared with those without roofs for both sampling lengths when propylene glycol was used as the preservative. For both the 8 day and 15 day sampling lengths there was no significant difference as determined by one-way ANOVA ($F(1,16) = 2.7684$, $p = .1156$) or ANOVA ($F(1,16) = 1.2491$, $p = .2802$) respectively. For those containing ethanol there was only a greater yield at the 15 day sampling length where there was a significant difference between DNA yield as determined by one-way ANOVA ($F(1,16) = 11.2758$, $p = .004$). There was no significant difference between DNA yields for the 8 days sampling as determined by one-way ANOVA ($F(1,16) = 1.3571$, $p = .2611$).

For control samples collected in water the DNA recovery ranged from 1.83 to 5.42ng/µl with a mean of 3.37ng/µl across all extractions meaning that on average ethanol and propylene glycol recovered 12 times more DNA than those stored in water. This fits with what was expected from the use of water as a sample collection solution. Samples collected in water were obviously degraded/decomposing when compared with propylene glycol when their appearance and odour were considered. It is also worth noting that samples collected in ethanol at longer time periods also showed some signs of degradation/decomposition when compared with propylene glycol.

In the 5 days after the 8-day traps were set up there were rain showers which would be expected to dilute the ethanol and propylene glycol within the traps without roofs. In fact, it was noted that some of the pitfall traps containing propylene glycol were full despite only

having 80 mL preservative solution added at set up (Katie Clark personal communication). Given that the DNA recovery from samples collected in water was poor one could expect the DNA recovery from the traps without roofs to be worse than for those with roofs due to rainfall dilution which was the case for the 15-day samples and for the 8-day propylene glycol samples. The pitfall traps containing ethanol were not full due to the evaporation of ethanol over the time course of the study.

In terms of evaporation of preservative, it was noted that almost all of the 21-day ethanol had evaporated by the time the samples were collected (Katie Clark personal communication). As field conditions were being observed the ethanol was not topped up during the 21 days as pitfall traps would not be topped up by Natural England staff after being set up. The other ethanol samples will have suffered from evaporation over the course of the sample collection, and this is a likely explanation for the decreasing yield of DNA recovered as sampling length and hence evaporation increased. Propylene glycol does not suffer from the same levels of evaporation as the ethanol as it does not evaporate to any significant degree which is likely one of the reasons as to why there is better yield of DNA recovered from these samples.

Sample ID	Description	DNA concentration (ng/µl)	A_{260}/A_{280}	DNA fragmentation (agarose gel)	Mean Cq Short Amplicon	Mean Cq Long Amplicon (Folmer)
E1_1	Ethanol 21d	11.10	2.11	High	16.7	20.62
E1_2		9.02	2.08	High	18.06	20.80
E1_3		9.56	2.06	High	17.02	20.36
E2_1	Ethanol 21d	3.47	2.10	High	18.78	20.97
E2_2		3.21	1.92	High	18.37	20.60
E2_3		3.15	1.96	High	18.52	21.34
E3_1	Ethanol 21d	11.30	2.09	High	18.89	21.47
E3_2		11.50	2.15	High	18.57	20.84
E3_3		8.96	2.11	High	16.98	20.26
E4_1	Ethanol 15d	51.80	2.08	High	20.33	21.47
E4_2		9.65	1.88	High	19.40	19.92
E4_3		18.00	2.15	High	17.44	20.09
E5_1	Ethanol 15d	5.94	1.91	High	17.26	20.99
E5_2		5.24	1.98	High	17.48	21.15
E5_3		5.83	2.06	High	17.17	20.92
E6_1	Ethanol 15d	7.18	2.06	High	17.89	22.13
E6_2		6.87	2.05	High	14.15	21.77
E6_3		9.66	2.04	High	17.42	22.62
E7_1	Ethanol 10d	9.63	2.12	High	16.52	20.09
E7_2		12.70	2.16	High	16.75	20.67
E7_3		12.50	2.02	High	16.98	20.41
E8_1	Ethanol 10d	28.70	2.00	Medium	18.94	20.95
E8_2		24.50	2.03	Medium	18.20	20.83

Sample ID	Description	DNA concentration (ng/µl)	A_{260}/A_{280}	DNA fragmentation (agarose gel)	Mean Cq Short Amplicon	Mean Cq Long Amplicon (Folmer)
E8_3		23.70	2.06	Medium	18.04	20.96
E9_1	Ethanol 10d	9.04	2.11	Medium	18.34	20.35
E9_2		9.72	2.09	Medium	18.24	20.14
E9_3		9.15	2.05	Medium	18.09	20.68
E10_1	Ethanol 8d	30.60	2.06	Medium	17.97	20.72
E10_2		32.30	2.04	Medium	18.03	21.42
E10_3		25.50	2.10	Medium	18.61	20.85
E11_1	Ethanol 8d	30.10	2.10	Medium	15.76	19.89
E11_2		26.70	2.12	Medium	15.44	20.29
E11_3		30.50	2.08	Medium	16.23	20.93
E12_1	Ethanol 8d	27.10	2.11	Medium	15.73	20.31
E12_2		32.70	2.12	Medium	15.57	20.04
E12_3		31.20	2.08	Medium	16.92	20.05
E13_1	Ethanol 3d	48.20	2.04	Low	18.21	19.92
E13_2		47.00	2.05	Low	20.40	20.29
E13_3		52.50	1.98	Low	20.61	21.12
E14_1	Ethanol 3d	23.40	2.04	Low	18.68	20.61
E14_2		32.80	2.11	Low	19.56	21.86
E14_3		41.60	2.01	Low	19.55	20.52
E15_1	Ethanol 3d	81.30	2.02	Low	17.35	20.23
E15_2		67.90	2.02	Low	19.19	19.86
E15_3		64.80	1.66	Low	17.99	19.65
ER4_1	Ethanol R d15	42.10	2.08	Medium	18.98	20.75
ER4_2		42.90	2.05	Medium	18.14	19.97
ER4_3		52.30	2.09	Medium	19.60	19.68
ER5_1	Ethanol R d15	25.70	2.10	Medium	17.79	21.68
ER5_2		17.30	2.11	Medium	17.94	21.09
ER5_3		22.10	2.08	Medium	19.21	21.43
ER6_1	Ethanol R d15	33.30	2.12	Medium	17.89	19.10
ER6_2		43.00	2.09	Medium	17.45	19.82
ER6_3		31.30	2.13	Medium	17.42	20.67
ER10_1	Ethanol R d8	22.40	2.13	Low	12.99	18.04
ER10_2		18.20	2.14	Low	14.48	18.60
ER10_3		21.50	2.15	Low	12.94	18.94
ER11_1	Ethanol R d8	75.40	2.05	Low	21.74	21.93
ER11_2		105.00	2.02	Low	20.60	21.42
ER11_3		77.20	2.04	Low	21.68	22.06
ER12_1	Ethanol R d8	21.20	2.11	Low	17.44	21.38

Sample ID	Description	DNA concentration (ng/µl)	A_{260}/A_{280}	DNA fragmentation (agarose gel)	Mean Cq Short Amplicon	Mean Cq Long Amplicon (Folmer)
ER12_2		21.50	2.04	Low	17.79	20.17
ER12_3		21.60	1.98	Low	16.71	20.60
EC1_1	Ethanol C d0	88.30	2.06	Low	16.19	21.00
EC1_2		99.80	2.08	Low	15.85	20.98
EC1_3		87.00	2.06	Low	15.83	21.78
EC2_1	Ethanol C d0	57.90	1.92	Low	18.19	23.13
EC2_2		73.60	1.94	Low	18.95	23.02
EC2_3		71.30	1.86	Low	17.86	22.17
EC3_1	Ethanol C d0	64.00	1.77	Low	16.55	21.46
EC3_2		60.40	1.70	Low	16.46	21.37
PG1_1	PG 21d	51.00	2.06	Medium	19.40	22.90
PG1_2		19.40	2.06	Medium	18.25	21.74
PG1_3		22.10	2.09	Medium	17.82	20.82
PG2_1	PG 21d	43.40	2.02	Medium	18.66	21.45
PG2_2		53.40	2.05	Medium	17.74	21.12
PG2_3		41.30	2.05	Medium	17.98	21.06
PG3_1	PG 21d	29.60	2.04	Medium	18.20	22.16
PG3_2		39.20	2.05	Medium	18.47	22.34
PG3_3		36.70	2.07	Medium	18.38	21.86
PG4_1	PG 15d	48.60	2.08	Medium	18.30	21.95
PG4_2		77.10	2.06	Medium	19.67	22.52
PG4_3		61.10	2.05	Medium	19.20	21.37
PG5_1	PG 15d	66.00	2.06	Medium	19.26	21.13
PG5_2		102.00	2.03	Medium	19.11	21.07
PG5_3		36.9.0	2.06	Medium	18.58	21.35
PG6_1	PG 15d	30.70	2.07	Medium	17.44	21.84
PG6_2		42.00	2.03	Medium	19.06	21.46
PG6_3		78.60	2.05	Medium	20.78	22.41
PG7_1	PG 10d	81.10	2.05	Medium	19.81	22.18
PG7_2		65.60	1.98	Medium	16.99	21.78
PG7_3		54.70	2.06	Medium	17.54	20.98
PG8_1	PG 10d	46.30	2.04	Medium	17.77	21.24
PG8_2		59.60	2.08	Medium	17.81	21.43
PG8_3		43.00	2.09	Medium	18.27	22.39
PG9_1	PG 10d	70.90	2.07	Medium	19.32	22.75
PG9_2		59.30	2.03	Medium	18.92	22.44
PG9_3		51.70	2.01	Medium	19.10	23.00
PG10_1	PG 8d	16.70	2.00	Medium	19.73	21.54
PG10_2		15.20	2.03	Medium	19.87	20.27
PG10_3		28.70	1.99	Medium	20.05	21.47
PG11_1	PG 8d	35.40	2.07	Medium	20.27	23.56

Sample ID	Description	DNA concentration (ng/µl)	A_{260}/A_{280}	DNA fragmentation (agarose gel)	Mean Cq Short Amplicon	Mean Cq Long Amplicon (Folmer)
PG11_2		29.50	2.02	Medium	20.44	23.78
PG11_3		21.50	2.01	Medium	20.00	22.74
PG12_1	PG 8d	56.40	2.05	Medium	18.18	20.32
PG12_2		93.90	2.03	Medium	18.23	21.36
PG12_3		54.40	2.05	Medium	17.50	21.89
PG13_1	PG 3d	86.00	1.91	Medium	20.15	21.04
PG13_2		84.10	2.06	Medium	17.70	21.60
PG13_3		79.90	2.12	Medium	17.81	21.39
PG14_1	PG 3d	24.80	1.84	Medium	18.39	22.57
PG14_2		50.90	2.10	Medium	18.06	22.04
PG14_3		78.00	2.04	Medium	18.60	22.15
PG15_1	PG 3d	71.70	1.98	Medium	19.43	21.48
PG15_2		85.40	1.99	Medium	19.18	20.77
PG15_3		76.40	1.98	Medium	19.97	22.23
PGR4_1	PG R 15d	59.70	2.07	Medium	17.06	19.91
PGR4_2		83.00	2.06	Medium	18.56	19.22
PGR4_3		123.00	2.05	Medium	17.85	20.79
PGR5_1	PG R 15d	50.90	2.03	Medium	18.76	22.42
PGR5_2		47.20	2.06	Medium	18.34	21.09
PGR5_3		46.50	2.05	Medium	16.87	21.34
PGR6_1	PG R 15d	67.50	2.03	Medium	19.81	22.54
PGR6_2		92.70	2.07	Medium	19.90	21.59
PGR6_3		88.50	2.04	Medium	20.70	21.68
PGR10_1	PG R 10d	103.00	2.01	Medium	19.28	24.59
PGR10_2		60.60	2.02	Medium	17.43	22.73
PGR10_3		46.50	1.85	Medium	21.50	22.06
PGR11_1	PG R 10d	66.50	2.07	Medium	18.77	23.34
PGR11_2		46.10	2.08	Medium	18.14	23.44
PGR11_3		34.30	2.10	Medium	17.46	20.79
PGR12_1	PG R 10d	41.70	2.07	Medium	18.16	21.80
PGR12_2		52.10	2.07	Medium	18.58	18.95
PGR12_3		62.50	2.02	Medium	19.50	21.30
PGC1_1	PG C d0	11.00	1.97	Medium	12.63	18.60
PGC1_2		12.70	2.16	Medium	13.70	18.64
PGC2_1	PG C d0	160.00	1.98	Medium	17.48	22.38
PGC2_2		133.00	1.98	Medium	17.76	20.98
PGC3_1	PGC d0	45.20	1.98	Medium	21.86	25.30
PGC3_2		35.00	2.03	Medium	21.25	23.49
PGC3_3		39.60	1.91	Medium	21.96	22.43
W2_1	Water d15	2.49	1.71	High	20.87	23.36
W2_2		1.89	1.29	High	21.95	24.06
W2_3		1.83	1.41	High	21.22	24.26
W4.4_1	Water d8	2.42	1.80	High	19.32	22.28
W4.4_2		2.57	1.79	High	18.70	21.58

Sample ID	Description	DNA concentration (ng/µl)	A_{260}/A_{280}	DNA fragmentation (agarose gel)	Mean Cq Short Amplicon	Mean Cq Long Amplicon (Folmer)
W4.4_3		2.32	1.74	High	18.33	21.80
W4_1	Water d8	4.97	1.77	High	17.81	22.56
W4_2		4.74	1.70	High	17.66	23.07
W4_3		5.18	1.92	High	16.42	22.84
W5_1	Water d8	2.35	1.57	High	N/A	N/A
W5_2		2.14	1.59	High	N/A	N/A
W5_3		2.11	1.60	High	N/A	N/A
W6_1	Water d8	5.16	1.76	High	18.46	24.19
W6_2		5.07	1.89	High	17.67	24.48
W6_3		5.42	1.76	High	18.25	25.14

Table 3. Sample information. Column headings left to right: sample ID; description; DNA concentration (ng/µl); $A_{260/280}$; DNA fragmentation (agarose gel); mean C_q short amplicon; mean C_q long amplicon (Folmer). Samples were collected in ethanol or propylene glycol (PG) for days 3, 8, 10, 15, 21. Samples were collected in water for 8 and 15 days. Traps with roofs to stop rain ingress were collected at days 8 and 15 (R), control samples (C) were collected at day zero. DNA concentration is that concentration of total DNA eluting from the extraction column. DNA was analysed on a 1.5% agarose gel and graded (by eye) into high (low molecular weight DNA/bottom of the gel), medium (middle of the gel), low levels (high molecular weight DNA/top of the gel) of DNA fragmentation. Each sample was DNA extracted in triplicate, each replicate DNA extraction was amplified in duplicate and these means of the duplicate PCR Cq values are recorded for each extracted DNA sample. Sample W5 did not amplify so was recorded as N/A as there was no Cq value.

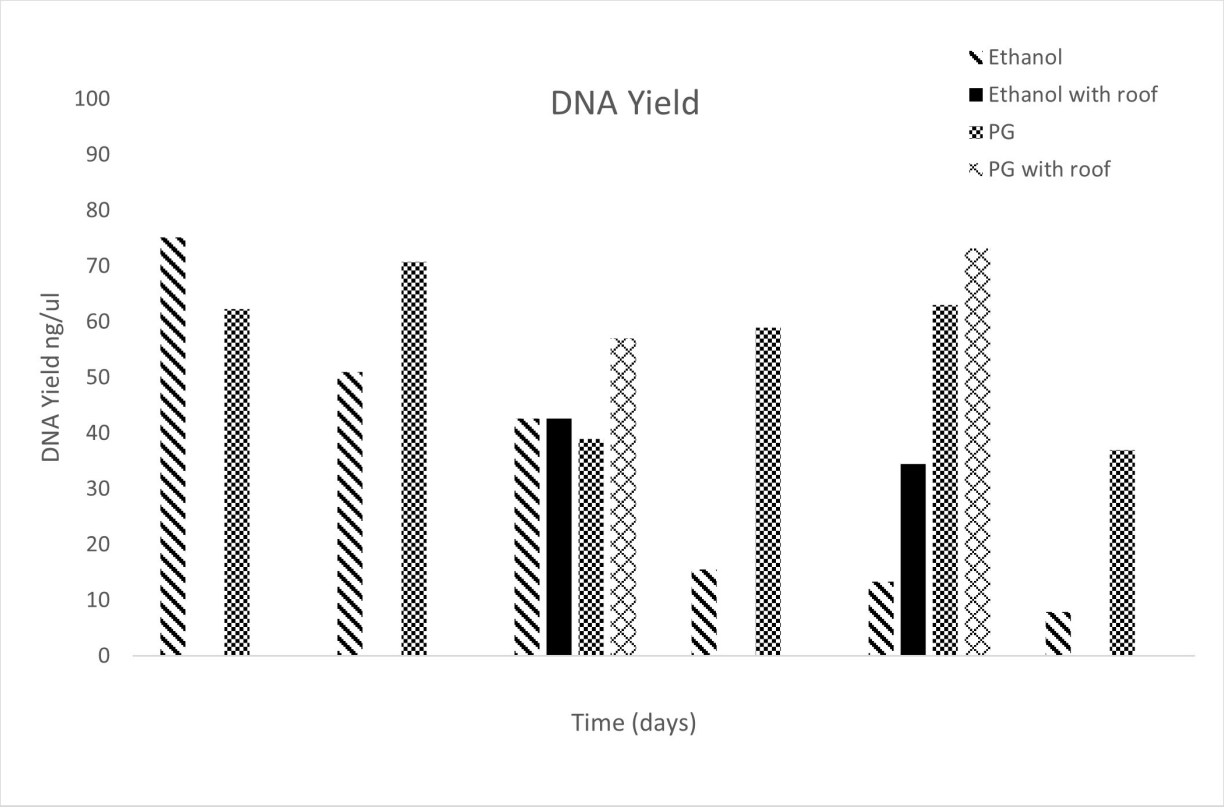

Figure 1. DNA Recovery. The mean DNA recoveries for all samples collected in either ethanol or propylene glycol at each timepoint (days). n = 3 pitfall trap samples per timepoint and 3 DNA extraction replicates per sample.

3.2 A_{260}/A_{280}.

The A_{260}/A_{280} values were measured by spectrophotometry and are shown in Table 3. This value is a measure of DNA purity where a ratio of around 1.8 and above is generally regarded as a DNA extract that is pure. Ratios lower than 1.6 suggest the presence of co-purified proteins or other contaminants that absorb at or around 280nm. The purity of all samples collected in both ethanol and propylene glycol were very good with only two ethanol stored samples having extracts (total of 4 extracts) with an A_{260}/A_{280} of less than 1.8 (EC3 and E15). Overall, the DNA extracted from both ethanol and propylene glycol stored samples had a mean A_{260}/A_{280} of 2.03 (Stdev of 0.15 and 0.05 respectively). The water stored control samples tended to have lower A_{260}/A_{280} ratios than the preserved samples (mean A_{260}/A_{280} of 1.68, Stdev: 0.17). This analysis suggests that neither the preservative type nor the length of sampling affects the purity of the DNA extracted, whereas those samples stored in the absence of preservative (and likely be degraded) are more likely to result in extracted DNA with the presence of contaminants.

3.3 DNA fragmentation

To visualise the level of DNA fragmentation (how well the DNA has been preserved) 1.5% Agarose/TAE gels were run to analyse each sample. The DNA fragmentation of recovered DNA is shown in Figure 2 and described in Table 3 as follows: High – most of the DNA is

present as shorter fragments of less than 500bp indicating substantial degradation; Medium – DNA is present as long and short fragments so shows partial degradation; and Low – most of the DNA is present as high molecular weight i.e. long fragments of DNA (bright band at the top of the lane) indicating only a small amount of DNA degradation. These high, medium, and low notations are shown graphically in Figures 3 and 4.

For storage in ethanol, higher molecular weight DNA indicative of undegraded material was the majority signal present in the control handpicked samples at T=0. Lower molecular weight fragments/less full-length DNA becomes more prevalent at the longer storage timepoints; compare E1-E6 with EC1-3 (Figure 2A). For ethanol these gels also highlight that less DNA was extracted at the longer timepoints - we were only able to run half the amounts of DNA on the gels due to the lower yields. For propylene glycol the fragmentation pattern looked more consistent for samples collected over the time course indicating that when sampling over longer time periods (more than 10 days), propylene glycol would be the best choice of preservative. Again, as expected samples collected in water show high amounts of DNA fragmentation (less DNA was run due to poor DNA extraction yields). The samples collected in the traps with roofs (less likely to be diluted by rainwater) showed no noticeable difference in fragmentation patterns when analysing the DNA by agarose gel electrophoresis. However, as noted previously pitfall traps with roofs allowed for higher DNA yields at both sampling times for propylene glycol due to the dilution effect of rainfall on the traps without roofs and for the 15-day sampling length for ethanol.

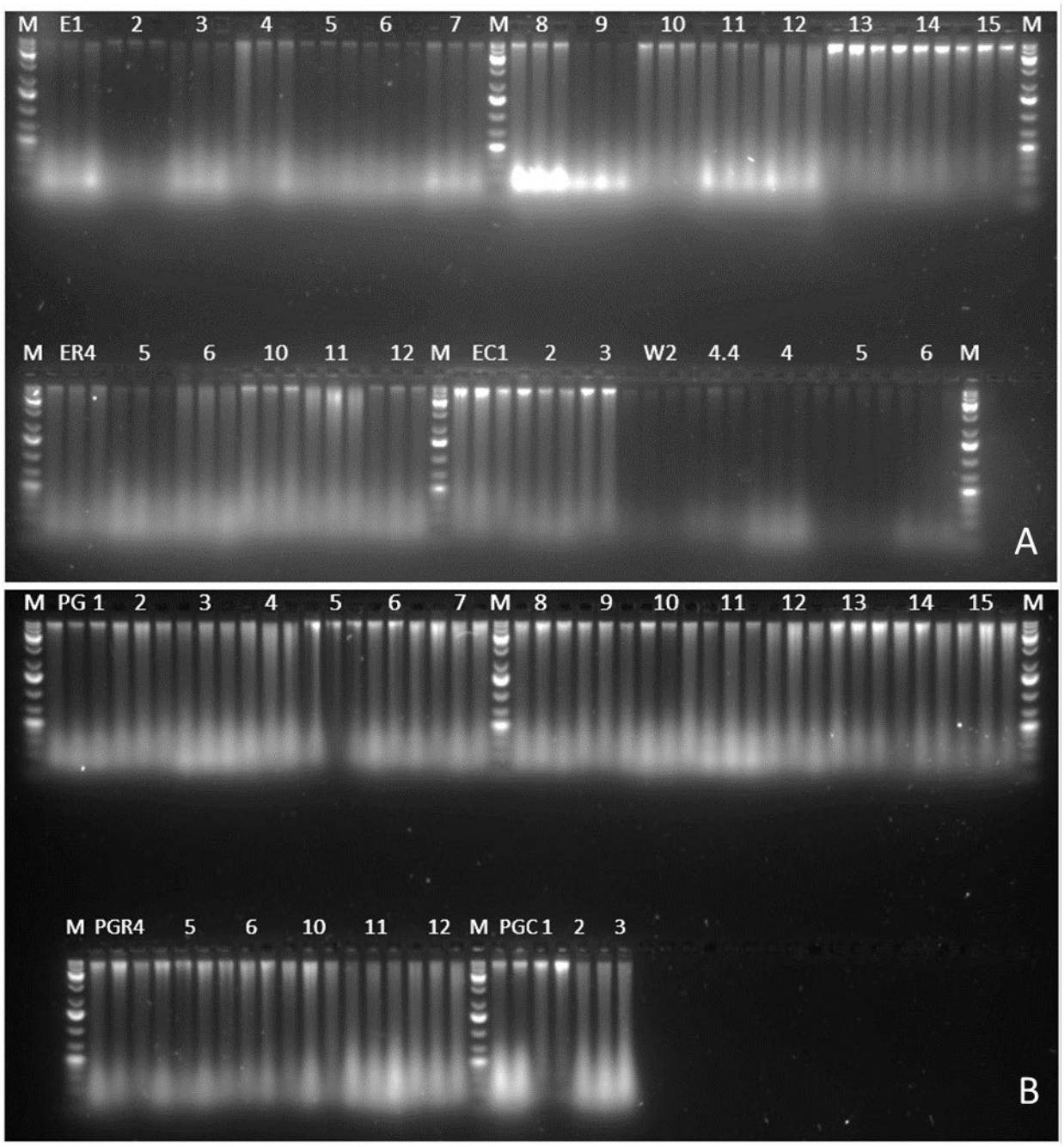

Figure 2A and 2B. DNA Fragmentation gels. Figure 2A: Top E1-E15. Lower panel ER4 4,5,6 and 10, 11, 12, EC 1, 2, 3 and water control 4-6. All lanes loaded with 150 ng of DNA except: E2_1,2,3 – 50 ng, E5_1,2,3 – 100 ng, E6_1,2,3 – 100 ng, E8_1,2,3 – 400 ng, W2_1,2,3 – 50 ng, W4.4_1,2,3 – 60 ng, W4_1,2,3 – 80 ng, W5_1,2,3 – 50 ng, W6_1,2,3 – 80 ng.

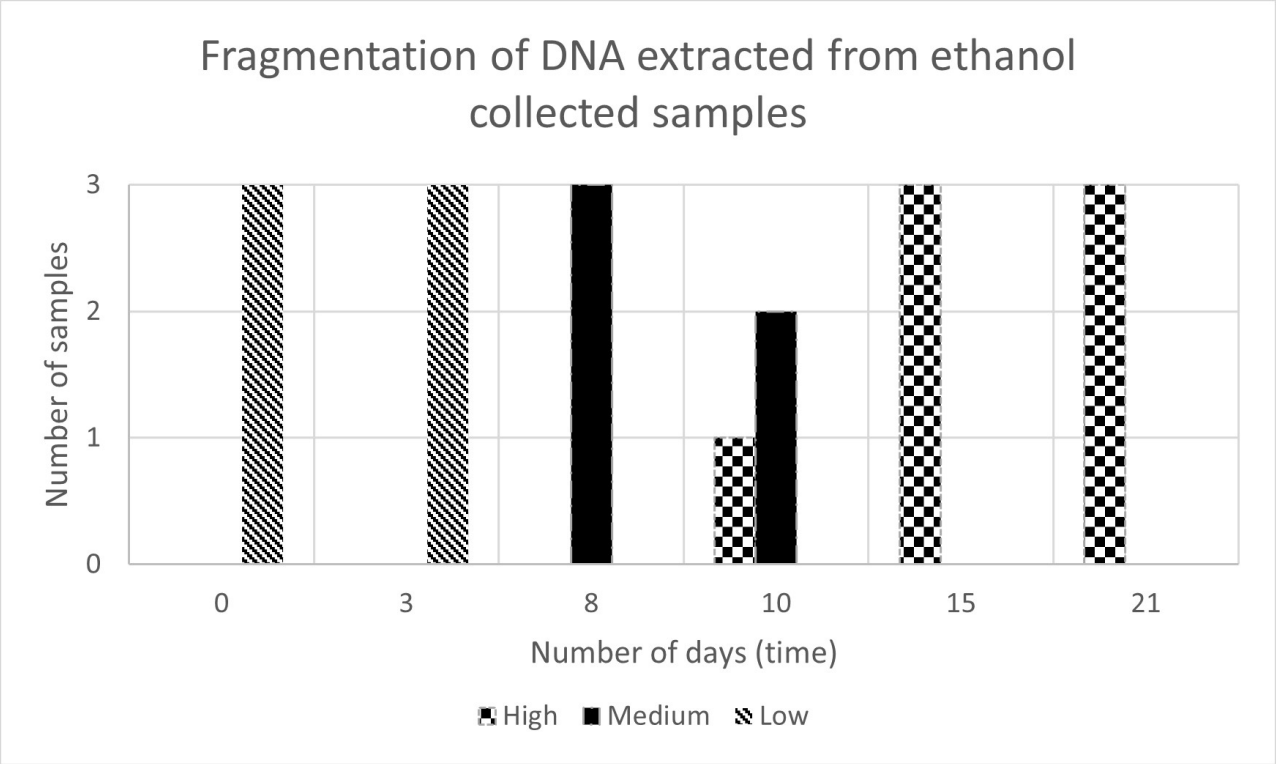

Figure 3. Ethanol sample DNA fragmentation. Graph illustrates that over time the level of fragmentation of the DNA increases.

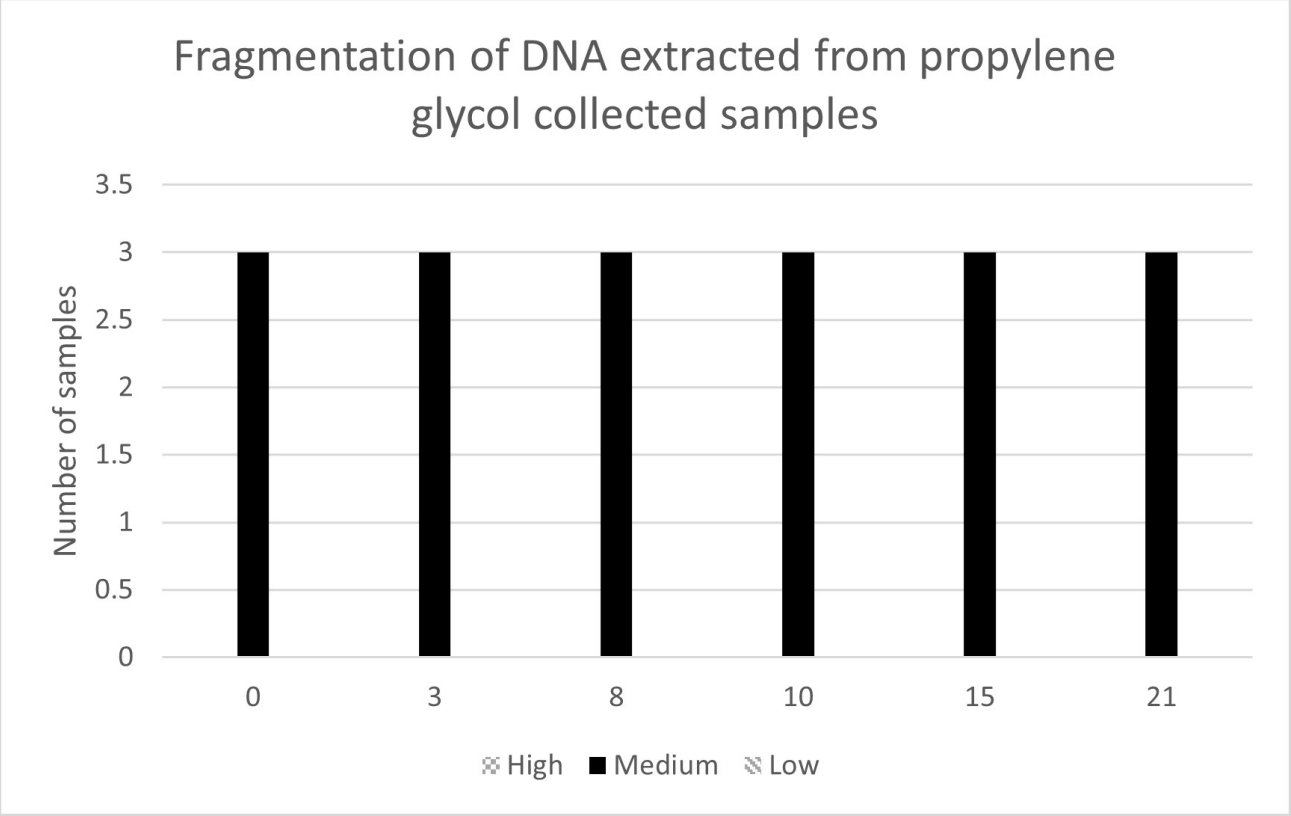

Figure 4. Propylene glycol sample DNA fragmentation. All samples showed medium fragmentation.

3.4 DNA barcoding PCR

Using PCR for the two amplicons of different sizes (205bp and 680bp) together with the observed size fractionation of fragments on agarose gels we can assess the suitability of a storage medium for DNA preservation. PCR using a standard amount of target DNA allows us to make a semi-quantitative assessment of the amount of DNA target present with relative samples. Cq values for each sample are shown in Table 3. The mean Cq values are shown in Figures 5 and 6.

Barcoding PCR amplicons were generated for all extracted DNA, except one sample collected in water (W5) which was not amplified with either set of primers, indicating a 100% success rate for both ethanol and propylene glycol collected samples. When compared with published studies (reviewed in Rees 2022) this study generally observed a higher PCR success rate, for ethanol and propylene glycol published PCR success rates varied from 60.5-100% or 65-100% respectively (Rees 2022).

The Cq value represents the PCR cycle at which amplification reaches a threshold of detectability, so is a measure of the amount of target DNA present in the starting sample. A lower Cq value would be consistent with a higher amount of non-degraded target DNA. The shorter PCR amplicon generally came up 3-4 Cq values before the longer for each preservative type, consistent with a greater amount of shorter or fragmented DNA in each sample. For both the ethanol and propylene glycol stored samples the Cq shifts over the sampling time course were modest. Samples stored in ethanol varied by 2.4 Cq values (short amplicon) or 1.5 Cq values (long amplicon) over the collection period (Figures 5 and 6). For those samples stored in propylene glycol the difference between the highest and lowest Cq was 1.9 Cq (short amplicon) and 0.47 (long amplicon). There was also a trend in the PCR Cq values demonstrating that the ethanol stored samples generally had a lower Cq value than the equivalent samples stored in propylene glycol for each of the amplicons tested. These differences are small and are generally within 1-2 Cq values indicating that for metabarcoding purposes both storage solutions would be suitable for downstream applications involving PCR. The presence of the trap roofs had no material effect on the Cq values that were generated, however rainfall was modest over the tine period that the samples were collected so may not be a true representation of possible sampling conditions. Overall, there was a tendency for the samples stored in ethanol to give a Cq value that was lower by 1-2 Cq values than the equivalent sample stored in propylene glycol.

At the longer sampling times propylene glycol gave much higher yields and more intact DNA than ethanol; however the Cq values were lower for ethanol even at these longer timepoints. This could suggest that residual propylene glycol could be inhibiting the PCR assays. A test of inhibition was therefore performed on all DNA extracts and, using the Cq values generated a one-way ANOVA test was performed for each sampling length between ethanol and propylene glycol samples. This found that in general there was no significant difference between samples collected in ethanol and propylene glycol. For example for the 10 day sampling there was no significant difference between Cq values as determined by one-way ANOVA ($F(1,4) = 0.8116$, $p = .3344$). In the two groups of samples

that did show a small significant difference as determined by one-way ANOVA (8 days: $F(1,4) = 0.7338$, $p = .0387$; and 15 days with roof: $F(1,4) = 1.0082$, $p = .0.0282$) this could likely be attributed to the intrinsic variation in the PCR caused by manual pipetting. Therefore, it is unlikely that the small differences in Cq values seen with ethanol and propylene glycol samples are due to the presence of residual propylene glycol in these samples. This is as expected as the propylene glycol samples were rinsed with ethanol prior to DNA extraction to remove the propylene glycol.

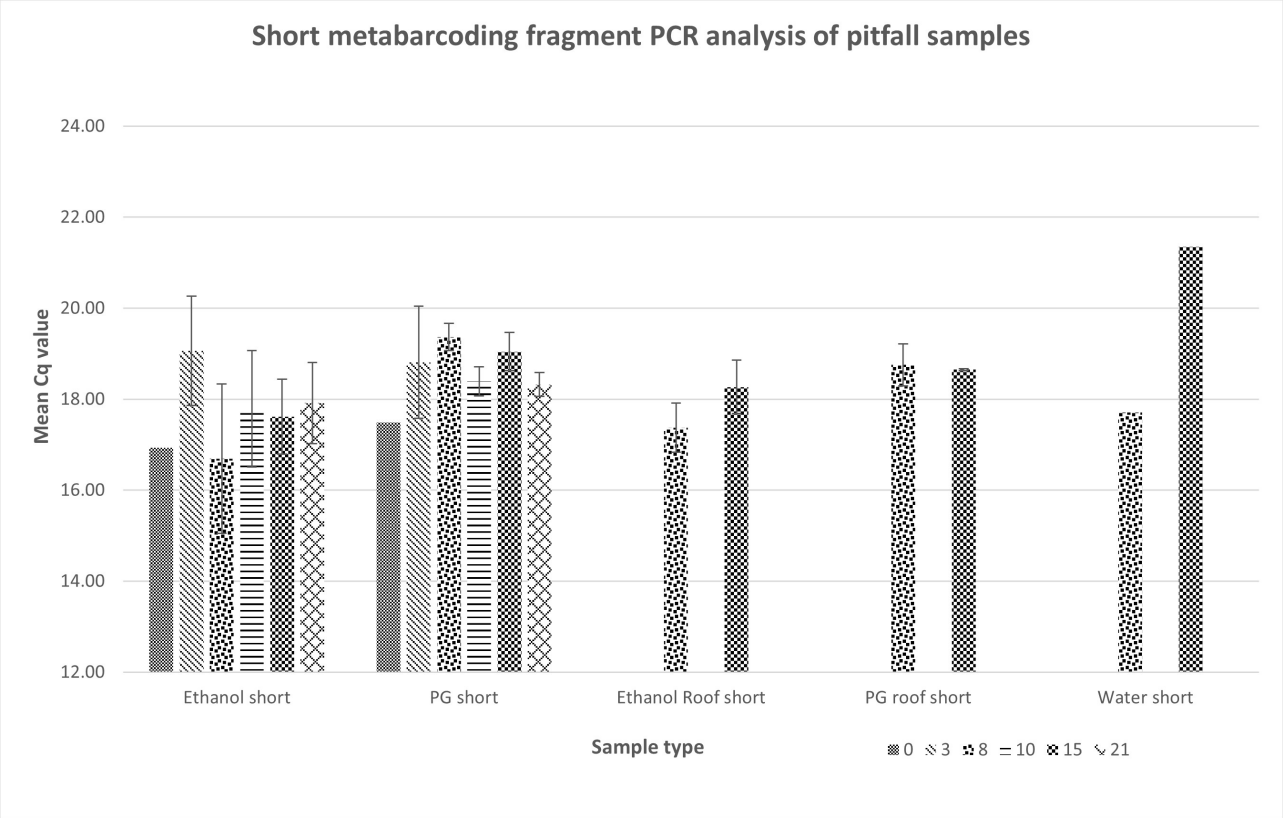

Figure 5. Short metabarcoding fragment PCR. Summary of all mean PCR data (mean of the triplicate pitfall trap samples – each extracted in triplicate and each extraction with duplicate PCR analysis i.e. n = 18); plotted are the mean Cq values of the PCR data taken from Table 3. 'Short' refers the length of the PCR barcoding amplicon (205bp); ethanol, PG and water refer to the preservative solution. 3, 8, 10, 15 and 21 refer to length of sampling (days).

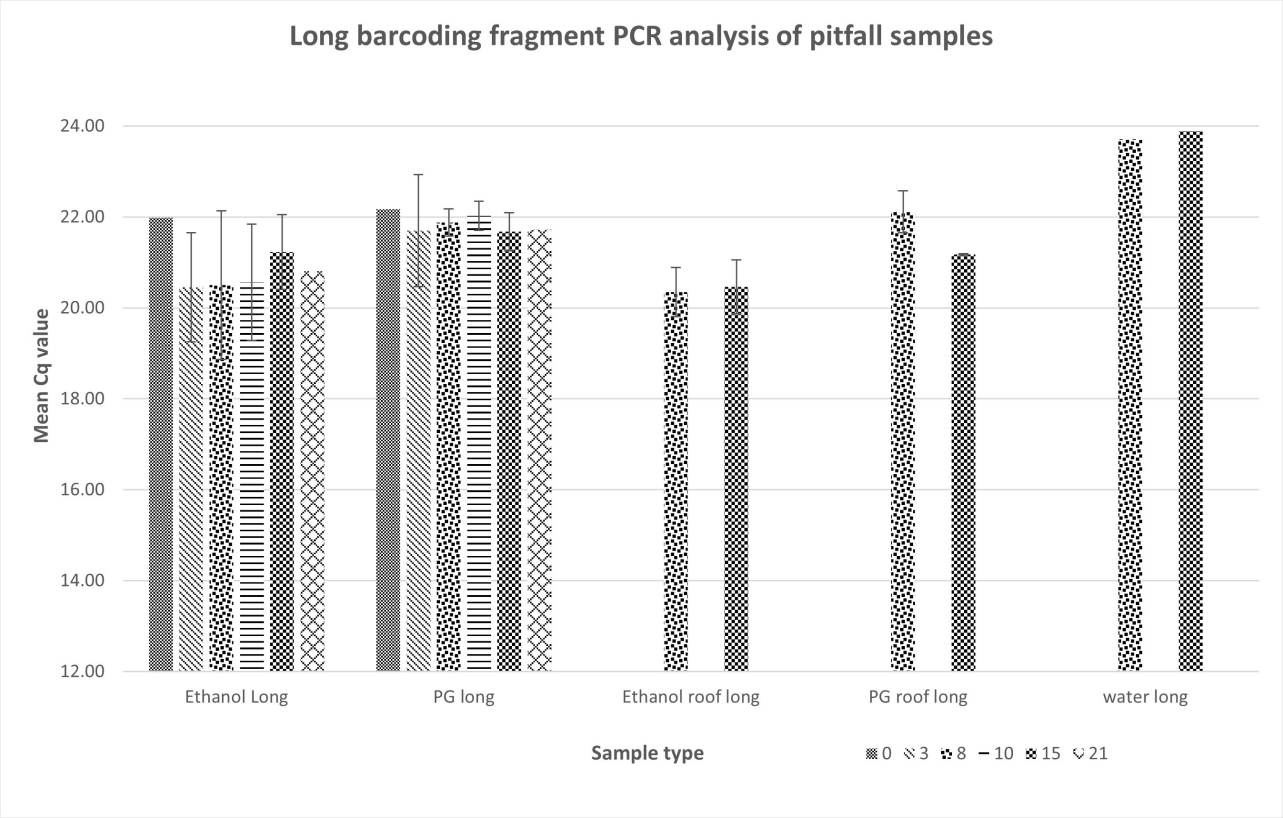

Figure 6. Long barcoding fragment PCR. Summary of all mean PCR data (mean of triplicate pitfall trap samples – each extracted in triplicate and each extraction with duplicate PCR analysis i.e. n = 18); plotted are the mean Cq values of the PCR data taken from Table 3. 'Long' refers the length of the PCR barcoding amplicon (680bp); ethanol, PG and water refer to the preservative solution. 3, 8, 10, 15 and 21 refer to length of sampling (days).

4. Concluding statements

We decided that further analysis i.e. sequencing of these samples was outside of the scope of this study. However, the yield and purity of DNA; alongside the success of PCR amplification with two sets of invertebrate specific primers; indicates that these pitfall trap samples would be amenable to invertebrate community analysis via metabarcoding. However, it is unknown whether the different preservatives and timepoints would lead to different community compositions upon sequencing. Further analysis using sequencing of mock communities would be required to determine whether, for example, the higher yields of DNA recovered from propylene glycol storage (at longer time points) leads to increased detection of low abundance species.

5. Recommendations

- Both ethanol and propylene glycol have proved appropriate for storage of intact amplifiable DNA for up to 21 days, and there is minimal loss in PCR sensitivity for

propylene glycol compared to ethanol. For metabarcoding approaches where the best DNA preservation method could assist in the identification of low abundance species (or prey species), the difference in PCR Cq recorded here are not likely to have any effect on species identification within complex mixtures of invertebrates. However, given the ease of use (cost, handling, toxicity) and the evaporation of ethanol, propylene glycol is recommended as a sampling reagent for invertebrates. Pitfall traps can be left in the field for up to 21 days, but if leaving for >10 days propylene glycol should be used and not ethanol.

- For longer sampling lengths (>15 days) the use of a roof allowed a greater yield of DNA to be recovered from the samples therefore the use of a pitfall trap with a roof is recommended for longer sampling times with the caveat that covers on pitfall traps can influence species composition (but not efficiency) and can preclude aerial capture so may not be suitable for some types of survey. The influence on species capture can be overcome using transparent roofs (Bell et al. 2014) as it is shading that seems to influence the catch. Also note that pan traps, which are designed for aerial faunas (e.g. flies, bees and wasps) utilise much higher volumes of fluid and have greater surface areas for evaporation and cannot, by design, have covers.
- Mock communities of invertebrates collected and stored in ethanol or propylene glycol could be subjected to metabarcoding and the results directly compared to determine if there are any major differences in the species identified. This would provide additional information on the suitability of propylene glycol as preservative solution of choice at the longer time frames.

6. References

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Appendix 1. Images of samples

Table 3. Pitfall trap sample images © Claire Baker and Steven Kane

 E1	 E2
 E3	 E4
 E5	 E6

E7

E8

E9

E10

E11

E12

E13

E14

E15

ER4

ER5

ER6

ER10

ER11

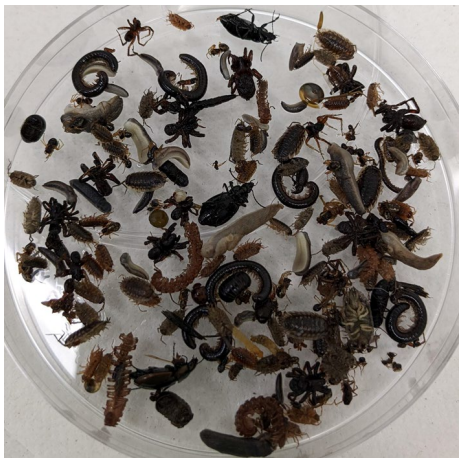
ER12

EC1

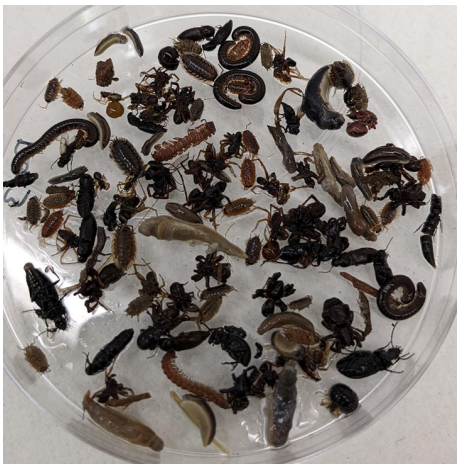
EC2

EC3

PG1



PG2

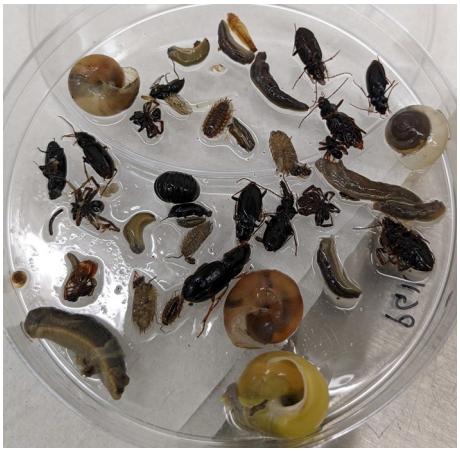


PG3

PG4

PG5

PG6

 PG7	 PG8
 PG9	 PG10
 PG11	 PG12

PG13

PG14

PG15

PGR4

PGR5

PGR6

 PGR10	 PGR11
 PGR12	 PGC1
 PGC2	 PGC3

W2

W4

W5

W6

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