Application of propylene glycol in DNA-based studies of invertebrates

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Abstract

High-throughput sequencing (HTS) studies on invertebrates commonly use ethanol as the main sample fixative (upon collection) and preservative (for storage and curation). However, alternative agents exist, which should not be automatically neglected when studies are newly designed. This review provides an overview of the application of propylene glycol (PG) in DNA-based studies of invertebrates, thus to stimulate an evidence-based discussion.

The use of PG in DNA-based studies of invertebrates is still limited (n = 79), but a steady increase has been visible since 2011. Most studies used PG as a fixative for passive trapping (73%) and performed Sanger sequencing (66%; e.g. DNA barcoding). More recently, HTS setups joined the field (11%). Terrestrial Coleoptera (30%) and Diptera (20%) were the most studied groups. Very often, information on the grade of PG used (75%) or storage conditions (duration, temperature) were lacking. This rendered direct comparisons of study results difficult, and highlight the need for further systematic studies on these subjects.

When compared to absolute ethanol, PG can be more widely and cheaply acquired (e.g. as an antifreeze, 13% of studies). It also enables longer trapping intervals, being especially relevant at remote or hard-to-reach places. Shipping of PG-conserved samples is regarded as risk-free and is authorised, pinpointing its potential for larger trapping programs or citizen science projects. Its property to retain flexibility of morphological characters as well as to lead to a reduced shrinkage effect was especially appraised by integrative study designs. Finally, the so far limited application of PG in the context of HTS showed promising results for short read amplicon sequencing and reduced representation methods. Knowledge of the influence of PG fixation and storage for long(er) read HTS setups is currently unavailable.

Given our review results and taking difficulties of direct methodological comparisons into account, future DNA-based studies of invertebrates should on a case-by-case basis critically scrutinise if the application of PG in their anticipated study design can be of benefit.

Key Words

alternative fixative, bioassessment, DNA integrity, DNA preservation, environmental monitoring

Introduction

DNA-based high-throughput sequencing (HTS) approaches such as DNA metabarcoding have lately revolutionised our ability to comparatively assess and monitor biodiversity over large geographical scales and at an unprecedented rate (Taberlet et al. 2012; Aylagas et al. 2016; Leese et al. 2018; Compson et al. 2020). Of particular relevance in practice are declining costs per sample, which are driven, among other things, by laboratory automation, comprehensive parallelisation of samples (i.e. multiplexing) and ever decreasing sequencing expenses (Leese et al. 2018). On the other hand, large-scale environmental programmes require a high number of appropriately conserved samples. Assuming that the number of samples to be processed within a scientific study or environmental program will continue to increase, also field costs per sample will account for an increasingly large financial share. It is therefore important to develop strategies to counterbalance the increasing costs of field
work, e.g. by reducing the time spent in the field, lowering hands-on times per sample by automation or adjusting collection and storage conditions (Holderegger et al. 2019; Syvank et al. 2018).

The fixative (or trapping / killing agent) is a crucial parameter for DNA-based studies as it has to secure the integrity of the DNA from the very beginning (see e.g. Stein et al. 2013). Simultaneously, its costs are directly linked to the number of samples to be processed and to the volumes used. It is not unusual for larger studies to require several hundred to thousand litres of fixative. Liu et al. (2020) identified lab grade ethanol (>95%) to be the fixative of choice in the majority of DNA metabarcoding studies conducted between 2015 and 2019. Yet, lab grade ethanol is very expensive, has a high volatility, is not always easy to obtain and due to its flammability can cause problems during sample transportation and shipping. Alternative fixatives exist but are not widely applied, e.g. due to misunderstandings of chemical properties, tradition or simply lack of knowledge and availability (Weeks Jr and McIntyre 1997; Thomas 2008; Nagy 2010; Stoeckle et al. 2010; Gossner et al. 2016; Malton et al. 2017).

As such an alternative, propylene glycol (or propane-1,2-diol; here further abbreviated as PG) has a number of characteristics that potentially render it beneficial in large-scale DNA-based HTS bioassessment and biomonitoring programmes: (i) low acquisition cost but high general availability, as it can be bought, for example, as low-budget antifreeze in specialist car dealers or as an additive from the cosmetic or food industry (additive E1520), (ii) non-toxicity (i.e. considered as a GRAS (generally regarded as safe) material), (iii) very low volatility, (iv) environmental safety, (v) risk-free transport of samples according to the regulations of the International Air Transport Association (IATA), and (vi) ability to ensure DNA integrity as well as to preserve most morphological characteristics (Thomas 2008; Nagy 2010).

Propylene glycol is a well-established agent in molecular cryobiology, notably used for the cryopreservation of sperms and cell cultures (Bank and Brockbank 1987; Hezavehei et al. 2018). For the study of invertebrates, it has been proposed as the chemical of choice in a standardised pitfall trap design for monitoring ground-active arthropod biodiversity (Brown and Matthews 2016; Höhbein and Conway 2018) and is applied as such within the North American National Ecological Observatory Network (NEON; Gibson et al. 2012; Hoekman et al. 2017) and the carabidologist community (Kotze et al. 2011). PG-based pitfall traps were also proposed as a standard and minimally disturbing method to investigate the subterranean fauna of the mesovoid shallow substratum (López and Oromi 2010; emptied every six months). Since 2001, PG fixation is applied by the Soybean Aphid Suction Trap Network (STN; Lagos-Kutz et al. 2020) for monitoring the ‘aerobiological soup’. In the context of environmental genomic studies, Matos-Maravi et al. (2019) proposed PG as the fixative of choice for mass samples originating from pitfall traps and flight interceptions, but neither tests nor metadata had been published along with this statement.

The aim of this review is to summarise the findings of studies investigating the application of PG for DNA-based analyses of invertebrates. We will distinguish its application as a fixative (during sample collection) and as a preservative (for sample storage and curation). The collated information should help to transfer the available knowledge to the wider community, stimulating an evidence-based discussion on how to further reduce costs for DNA-conform sample collection and curation in larger environmental programmes by exploring alternative fixation and preservation agents.

**Material and methods**

**Literature search**

A topical core literature research was conducted on 07.10.2020 within the ISI Web of Science (WoS) and screening ‘all databases’. The following search strings were investigated: (1) “propylene glycol” AND “DNA” AND “invertebrate*”, (2) “propylene glycol” AND “DNA” AND “insect”, (3) “propylene glycol” AND “DNA” AND “invertebrate*” and (4) “propylene glycol” AND “DNA” AND “insect”. On the very same day, and because the WoS search only will detect literature records which are ISI-listed and only will retrieve hits in case the search string terms appear in the title, abstract, topic or as keywords, a complementing Google Scholar (GS) search was performed (as e.g. proposed by Piasecki et al. 2018). The GS search enables in-text searches and will also retrieve grey literature records such as field, laboratory and shipping protocols, pre-prints, theses and agency reports. The same four search strings were entered, selecting the ‘exclude patents’ option in GS. The keyword ‘sperm*’ was excluded in all searches as PG is frequently applied during the cryopreservation of sperms which would have resulted in a very high number of irrelevant hits. The key word ‘invertebrate*’ was excluded in the search strings (2) and (4) to not again screen insect literature records retrieved from (1) and (3).

The four search strings yielded the following number of literature records within the ISI WoS: (1) 20, (2) 8, (3) 0 and (4) 1. From those, only records which transparently stated the application of PG during specimen handling have been considered, resulting in a total of only 12 studies. Google Scholar search strings yielded the following constant number of literature records, regardless of whether the search was performed via two different IP-addresses in two countries (Luxembourg and Germany), within two different Google profiles, using a private browser window or being logged on/off from personal Google profiles: (1) 509, (2) 1930, (3) 23 and (4) 432.

**Results**

The majority of studies had to be excluded, a) because they used PG as a trapping material for invertebrates but only...
cited literature referring to the application of DNA-based tools, or b) as they referred to yeast species molecularly analysed. Furthermore, reviews or studies which only dealt with the DNA analysis of e.g. parasites or gut contents of invertebrates were omitted, but will be discussed. Doctoral and master theses which were subsequently published as scientific articles were counted as a single entry.

A total of 79 publications was retrieved (Table 1, Fig. 1). Although the restricted number of literature records obtained exemplifies that the number of studies using PG as a fixative or preservative for subsequent molecular DNA-based analysis of invertebrates is not widely established (but an increase can be noted), still some patterns can be inferred. The analysed studies almost exclusively investigated terrestrial taxa, with Robinson et al. (2020; freshwater bulk sample) and Cordero et al. (2017; aquatic insects) being notable exceptions. The first studies using PG for DNA-based analysis of invertebrates originate from 2003 and 2005. Two of them focussed on honey bees (Rubink et al. 2003; Coulson et al. 2005) while Carter (2003) performed a comprehensive study on ‘The effects of preservation and conservation treatments on the DNA of museum invertebrate fluid preserved collections’ using a terrestrial isopod species as reference. Those studies were accompanied by early publications on arachnids (Vink et al. 2005) and hemipterans (Scott et al. 2007). From today’s perspective, studies focussing on Coleoptera (n = 24 studies, 30%) and Diptera (20%) dominated the dataset (Fig. 1). Although Diptera was the second largest of the individual groups retrieved, the first study was only published in 2012 and most of the records originate from the same team of authors.

In the majority of study designs, PG was used as a fixative for passive trapping (n = 53, 73%), and less frequently as a fixative upon manual collection of living specimens (32%). Either food-/laboratory-grade PG (14%; Sigma-Aldrich, Ajax FineChem, Neogen, Herrlan-PSM, Old World Industries, Better World Manufacturing, ClassiKool Ltd.) or PG-based antifreezes (13%; Lowtox, Absolute Zéro RV Waterline, Sierra, Uni-Gard) were used, but in 75% of all studies no further chemical properties or customer specifications were provided. PG concentrations were in most cases higher than 95% (for 70% of study designs), in fewer instances between 50–75% (16% study designs) or below 50% (11% study designs). Information on storage conditions for PG-preserved specimens was also very scarce: 27% of studies did not report any storage duration and for 41% of studies no storage temperatures were provided. Otherwise, PG-preserved specimens were stored for quite variable time spans, i.e. for more than half a year (10%), 1–6 months (10%), 1–4 weeks (20%), below 1 week (17%) or even for shipping only (17%, variable duration). If information was provided, specimens most often were stored at RT (28%), less frequently frozen (17%) or refrigerated (10%). Sanger sequencing was the most frequent evaluation method (66%), followed by PCR-based analyses (16%), HTS (11%) and microsatellite genotyping (9%). Studies performing two conceptual approaches, e.g. Sanger sequencing of COI and COI metabarcoding, were included in both categories.

Figure 1. Cumulated number of studies which have used propylene glycol either as a fixative or preservative in the DNA-based analysis of invertebrates. Arranged by the taxonomic groups in focus. In cases where several of the indicated groups were targeted, the study was placed into the category “others”. N = 79, as of 07.10.2020.

Discussion

Application of propylene glycol as a fixative

Our results indicate that PG is widely applied as a fixative in a variety of passive trapping methods (e.g. pan trap, funnel trap, aerial pitfall trap; baited and unbaited) and for various organism groups (mainly beetles and flies, but also spiders, bees and aphids). Likewise, actively collected single specimens or – in a few cases – invertebrate bulk samples were fixed with PG (e.g. Bowser et al. 2017, 2019; Cordero et al. 2017; Jusino et al. 2019; Robinson et al. 2020; Liu et al. 2020). This taxonomically widespread application mimics the use of PG in traditional non-DNA-based research and application domains (primarily pitfall trapping; see Hohbein and Conway 2018) now entering the molecular field. Research communities around aphids, spiders, carabids and tephritid fruit flies contributed most of the studies.

Amongst others, passive trapping intervals are determined by the accessibility of the sampling location, the volume of the trapping containers, the evaporation rate of the fixative and local environmental parameters such as humidity, temperature, rainfall or UV-exposure. In case of excessive heat and high temperatures (arid and hot), traps with ethanol or (salted) water frequently dry out and either have to be visited and re-filled more frequently, or short(er) trapping periods have to be chosen. The evaporation rate of PG is >500-times lower than for ethanol (Moreau et al. 2013). As such a low volatile agent, PG-equipped traps can retain their volumes more or less constant over several weeks to months (Hohbein and Conway 2018). However, long-term UV exposition may decompose PG into water, acetone and 2-propanol (i.e. isopropanol) (Nakahama et al. 2019). In interaction with its hygroscopic nature, even apparently large fixative volumes at the end of a long trapping period under summer conditions might contain a
Table 1. Overview of studies which have used propylene glycol either as a fixative or preservative for the DNA-based analysis of invertebrates. RT = room temperature; PG = propylene glycol; n.a. = not applicable or not available. Studies found by both the ISI Web of Science and Google Scholar searches, are marked in bold. All others were only detected by the latter search engine. N = 79, as of 07.10.2020.

| Study | Year | Taxon | DNA-based approach | PG specifics | Fixation step | Preservation step | Central outcomes |
|-------|------|-------|---------------------|-------------|---------------|------------------|-----------------|
| Carter (2003) | 2003 | Lycosa (Psocida) | PCR-based visualisation (16S, 18S) | Propylene glycol | specimens directly placed in pure PG | 12 months (RT) | double-stranded DNA profiles of PG-conserved specimens were sufficient for PCR, but likely not adequate for long-term storage of museum samples |
| Rubink et al. (2003) | 2003 | Hymenoptera (Apidae) | Microsatellite and CytoB fragment analyses | Low-toxicity antifreeze (Lowtox, Prestone Inc., Danbury, CT) | specimens directly placed in pure PG | 5, 20 or 90 days (20 °C or 40 °C) | up to 4 months (4-6 °C) | nuclear and mtDNA were amenable even at the most extreme conditions (90 days, 40 °C), although a slightly decreasing trend was observed |
| Cordson et al. (2005) | 2005 | Hymenoptera (Apidae) | Cyth, 16S and COI fragment analysis | Propylene glycol | baited aerial pitfall trap with 50% PG and soap | ca. weekly for 8 months | 95% ethanol (4 °C) | well preserved for molecular analysis |
| Vink et al. (2005) | 2005 | Arachnida (Aranea, Scorpionida) | Actin and COI fragment analysis | Propylene glycol (99.5%+ laboratory grade, Sigma-Aldrich) | specimens directly placed in pure PG | 6 weeks (40 °C, 19-24 °C, 2-4 °C, -20 °C or -40 °C; dark) | 95% ethanol (4 °C) | 1 day | PS (and RNA) better preserved nuclear and mtDNA than ethanol at various concentrations and in different study designs: mtDNA successfully amplified under most extreme conditions (6 weeks, 40 °C); ncDNA for small soft-bodied species only at room temperature or lower, and for large heavily sclerotized species at 2-4 °C or lower |
| Hendriksen (2006) | 2006 | Arachnida (Aranea) | Sanger sequencing of COI and 28S | Propylene glycol | pan trap with 1:1 PG and 100% ethanol | each 2 weeks for 2 months | 100% ethanol, than 80% ethanol (20 °C) | n.a. | well preserved for morphological and molecular analyses |
| Scott et al. (2007) | 2007 | Hymenoptera (Aleyroidea) | Sanger sequencing of COI | Propylene glycol | specimens directly placed in pure PG | n.a. | n.a. | well preserved for morphological and molecular analyses |
| Galligo and Galán (2008) | 2008 | Coleoptera (Curculionidae) | Sanger sequencing of COI | Propylene glycol | funnel trap with pure PG | 1 week | absolute ethanol | n.a. | well preserved for morphological and molecular analyses |
| Villacorta et al. (2008) | 2008 | Crustacea (Amphipoda) | Sanger sequencing of COI, COII and H3 | Propylene glycol | baited pitfall trap with PG | several weeks to months | ethanol | n.a. | well preserved for morphological and molecular analyses |
| Castalanelli et al. (2010) | 2010 | Coleoptera | Sanger sequencing of mtDNA, single and multi-copy ncDNA genes | Propylene glycol | specimens directly placed in 20% PG | n.a. | ethanol | n.a. | very fast DNA isolation (ranging from 2-20 minutes); well preserved for parallel morphological and genetic analyses; specimens stored in 20% PG and ethanol did not differ from specimens stored in other preservatives |
| Horn (2010) | 2010 | Hymenoptera (Anthophila) | Sanger sequencing of COI | Propylene glycol | pan trap with 75% PG | each two weeks | 95% ethanol (refrigerator) | n.a. | specimens suitable for barcoding |
| Malambres Olarte (2010) | 2010 | Arachnida (Arana) | Sanger sequencing of COI | Mono-propylene glycol | pitfall trap with pure PG | 2 weeks over 3 months | 95% ethanol (20 °C) | n.a. | well preserved for morphological and molecular analyses |
| Shoda-Kagaya et al. (2010) | 2010 | Coleoptera (Curculionidae) | Microsatellite genotyping | Propylene glycol | pheromone-baited trap with PG | n.a. | 90.5% ethanol (40 °C) | n.a. | specimens suitable for microsatellite analysis |
| Scodda et al. (2010) | 2010 | Arachnida (Arane) | Sanger sequencing of COI and restriction site analysis | Propylene glycol | pitfall trap with 20% PG | 1 or 2 weeks | 90% ethanol | months | well preserved for morphological and molecular analyses |
| Boyer et al. (2011) | 2011 | Anelida (Oligochaeta) | Sanger sequencing of COI and 16S | Propylene glycol | specimens directly placed in 98% ethanol | n.a. | PG | n.a. | well preserved for morphological and molecular analyses |
| Stevens et al. (2011) | 2011 | Coleoptera (Tenebrionidae, Bostrichidae) | Arginine kinase fragment analysis | 99.5% Propylene glycol (Sigma- Aldrich Inc, St. Louis, MO) | specimens directly placed in PG (100%, 80%, 50%, diluted with PBS, with/without Triton-X 100) | 3, 7 or 14 days (30 °C) | absolute ethanol (Sigma- Aldrich, molecular grade) (40 °C) | n.a. | treatments with specimens stored in mixtures containing PG produced significantly less successful PCR results. PCR success was higher for specimens stored in pure PG than for 80% PG |
| Castalanelli et al. (2012) | 2012 | Coleoptera (Dermestidae) | Sanger sequencing of COI, Cyth and 18S | Propylene glycol | baited lure trap with 20% PG | two months | rinsed with sterile water, rinsed with 70% ethanol and stored in 95% ethanol | n.a. (20 °C) | well preserved for morphological and molecular analyses |
| Study                          | Year   | Taxon               | DNA-based approach             | PLT specifications | Fixation step | Preservation step | Central outcomes                                      |
|-------------------------------|--------|---------------------|-------------------------------|-------------------|---------------|-------------------|-------------------------------------------------------|
| Gibson et al. (2012)          | 2012   | Coleoptera (Carabidae) | Sanger sequencing of COI      | Propylene glycol  | pitfall trap with 66% PG | 1 week | 75% ethanol, or rimmed in water and stored in 95% ethanol (20 °C) | n.a. well preserved for morphological and molecular analyses |
| Gruber et al. (2012)          | 2012   | Hymenoptera (Formicidae) | Sanger sequencing of COI      | Propylene glycol  | pitfall trap with 33% PG | 1 day | 95% ethanol (4 °C) | n.a. well preserved for morphological and molecular analyses |
| Knee et al. (2012, Knee (2012) | 2012   | Formicidae          | Sanger sequencing of COI      | Propylene glycol  | baited Lindgren funnel trap with PG | <2 weeks | 95% ethanol (20 °C) | n.a. well preserved for morphological and molecular analyses |
| Pelletier et al. (2012)       | 2012   | Hemiptera (Acanthosomatidae) | Sanger sequencing of COI      | Propylene glycol  | trap with 50% PG with Bitrex and soap | each 2–3 days for 2 months | 50% PG (4 °C) up to 1 week | PG concentration checked in the field remained in the range 40–60%; collected material mostly suitable for morphological analysis, barcoding and RNA virus detection; although higher concentrations yielded better results |
| Renaud et al. (2012, Renaud (2012) | 2012   | Diptera (Muscidae) | Sanger sequencing of COI      | Propylene glycol  | pan trap with 33% PG and soap | 3–4 days | n.a. | n.a. handling of PG was problematic because treated as hazardous waste and forbidden to dispose in local septic systems; specimens suitable for barcoding and morphological analysis |
| Schütze et al. (2012)         | 2012   | Diptera (Calliphoridae, Fanniidae, Muscidae, Tephritidae) | Sanger sequencing of COI      | Propylene glycol  | lure-based hanging trap with pure PG | n.a. | assumably pure PG | n.a. easy transport of samples; >90% of specimens morphologically characterised and barcoded |
| Vélez et al. (2012)           | 2012   | Myriopoda (Chilopoda) | Sanger sequencing of COI and 16S | Propylene glycol  | specimens directly placed in 95% or 75–80% ethanol | n.a. | PG, than 90% ethanol | n.a. effective shipping of samples; well preserved for molecular analysis |
| Ferro and Park (2013)         | 2013   | Coleoptera (Carabidae, Staphylinidae) | Sanger sequencing of COI      | Propylene glycol  | specimens directly placed in 100% ethanol | 2 days | 20%, 40%, 60%, 80% and pure PG (21 °C) up to 6 months | positive PCR or sequencing results were obtained in all cases except for 20% PG |
| Krosch et al. (2013)          | 2013   | Diptera (Tephritidae) | Microsatellite genotyping     | Propylene glycol  | insecticide-based hanging trap with PG | n.a. | n.a. | effective shipping of samples; well preserved for morphological and molecular analyses |
| Moreau et al. (2013)          | 2013   | Hymenoptera (Formicidae) | Long-wavelength rhodopsin and COI fragment analysis | Propylene glycol  | specimens directly placed in pure PG | either remaining in PG for up to 10 months or transferred into 95% ethanol after 6 months (6+4 months storage time) | PG and ethanol allowed for the highest PCR success rates. PG-preserved samples showed comparatively high DNA concentrations even after 10 months |
| Sekes and Stockbridge (2013), Stockbridge (2013) | 2013   | Mecoptera | Sanger sequencing of COI      | Propylene glycol based antifreeze (Sierra brand) | pitfall trap with PG | two weeks | 100% ethanol (-70F) | n.a. well preserved for molecular analysis |
| Sim (2013)                    | 2013   | Arachnida (Araneae) | Sanger sequencing of COI, ITS and NDI | Propylene glycol  | pitfall and pan trap with 50% PG | 3–4 days for 2 weeks | 95% ethanol (4 °C) | n.a. well preserved for morphological and molecular analyses |
| Boykin et al. (2014)          | 2014   | Diptera (Tephritidae) | Sanger sequencing of COI, NAD4–3', CAD, period, ITS1 and ITS2 | Propylene glycol  | insecticide-based hanging trap with PG | variable | 100% ethanol | n.a. effective shipping of samples; well preserved for morphological and molecular analyses |
| Endo et al. (2014)            | 2014   | Coleoptera (Carabidae), Myriopoda, Colembola | Sanger sequencing of COI, ITS1 and ANT | Propylene glycol  | pitfall trap with PGethanol (1:1) | n.a. | 100% molecular-grade ethanol | n.a. well preserved for molecular analysis |
| Gomez (2014)                  | 2014   | Coleoptera (Carabidae) | Sanger sequencing of COI, CAD and 28S | Propylene glycol  | pan trap with PG | n.a. | n.a. suitable for all molecular investigations; however, higher amplification success when PG-fixed specimens were dry-pinned or transferred to 95% ethanol within 1–2 weeks |
| Chuvinjukul et al. (2015)     | 2015   | Diptera (Tephritidae) | Sanger sequencing of ITS1     | Propylene glycol  | specimens directly placed in 95% ethanol | n.a. | pure PG | n.a. effective shipping of samples; well preserved for molecular analysis |
| Fountain et al. (2015)        | 2015   | Coleoptera (Curculionidae) | Sanger sequencing of COI, CyH and ITS2 | Propylene glycol  | specimens directly placed in PG | n.a. | 95% ethanol (-20 °C) | n.a. well preserved for molecular analysis |
| Hase and Zieliske (2015)      | 2015   | Gastropoda (Caenogastropoda) | Sanger sequencing of COI, 16S and ITS2 | Propylene glycol  | specimens directly placed in 70% ethanol | n.a. | PG, than 96% ethanol | PG preservation only for shipping effective shipping of samples; well preserved for morphological and molecular analyses |
| Höfer et al. (2015)           | 2015   | Arachnida (Araneae) | Sanger sequencing of COI      | Propylene glycol (technical grade, Herrfan-PSM) | specimens directly placed in pure, 90% or 50% PG | 1.2 and 4 weeks (refrigerator) | non-denatured 96% ethanol | n.a. successful barcoding under all conditions, but results potentially indicate a negative effect of water intrusion on PG-preserved specimens |
| Study                           | Year    | Taxon                  | DNA-based approach | PCR specifications | Fixation step | Preservation step | Central outcomes                                                                 |
|--------------------------------|---------|------------------------|--------------------|--------------------|---------------|------------------|-----------------------------------------------------------------------------------|
| LeBlanc et al. (2015)          | 2015    | Diptera (Tephritidae)  | Sanger sequencing  | Propylene glycol (Better World Manufacturing, Fresno, CA) | baited Lindgren funnel trap with 25% PG | 95% ethanol (freezer) | well preserved for morphological and molecular analyses                           |
| Ragnar-Jones et al. (2015)     | 2015    | Coleoptera (Curculionidae) | Sanger sequencing of COI and 28S | Pink marine or recreational vehicle antifreeze (not for automobiles) | ethanolt-baited Lindgren funnel trap with PG | weekly or bi-weekly | 100% ethanol (refrigerator) | well preserved for molecular analysis; part of specimens collected in PG-based antifreeze according to official monitoring guidelines (Seybold et al. 2013) |
| Sanchez Garcia et al. (2015), Sanchez Garcia (2015) | 2015    | Coleoptera (Curculionidae) | Sanger sequencing of COI and COII | Propylene glycol | n.a. | n.a. | n.a. | well preserved for morphological and molecular analyses |
| Smith and Cognato (2015)       | 2015    | Coleoptera (Curculionidae) | Sanger sequencing of COI and 28S | Propylene glycol | n.a. | n.a. | n.a. | well preserved for morphological and molecular analyses |
| Steinhager et al. (2015)       | 2015    | Coleoptera (Curculionidae) | Arginine kinase fragment analysis | 99% extra pure PG (Fisher) and low-toxicity antifreeze (Lowtox, Prestone Inc.) | specimens directly placed in pure PG or PG-based antifreeze | 2 or 7 days | very high qPCR success rates, no matter which storage conditions were used |
| Borges et al. (2016)           | 2016    | Coleoptera (Zopheridae) | Sanger sequencing of COI, COII, RNA-Leu gene and EF1α | Propylene glycol | pitfall trap with pure PG | 100% ethanol or acetonitrile (refrigerator) | n.a. | well preserved for morphological and molecular analyses |
| Eigenbrode et al. (2016)       | 2016    | Hemiptera (Aphididae)  | Microsatellite genotyping | Propylene glycol | pan trap with PG | 95% ethanol (−60 °C) | n.a. | well preserved for microsatellite analysis |
| Liu (2016)                     | 2016    | Diptera (Drosophilidae) | Microsatellite genotyping | Propylene glycol | baited bottle trap with PG | 95% ethanol (−60 °C) | n.a. | well preserved for morphological and microsatellite analyses |
| Patrick et al. (2016)          | 2016    | Diptera (Calliphoridae, Fanniidae, Muscidae, Tephritidae) | High-quality genomic DNA for HTS approaches | 99.5% propylene glycol (Sigma-Aldrich) | specimens directly placed in pure PG | 1, 8, 13, 14 or 15 days (4 °C or -20 °C) | PG, 97–100% ethanol and AL buffer yielded high-quality genomic DNA, whereas RNA-free water, buffer AE and PBS failed. DNA concentration in ethanol was significantly higher at both temperatures |
| Postlethwaite (2016)           | 2016    | Hymenoptera (Anthophila) | Sanger sequencing of COI | Propylene glycol | pitfall trap with pure PG | days to few months | PG until pinning | n.a. | effective shipping of samples; well preserved for morphological and molecular analyses |
| Rebideau et al. (2016)         | 2016    | Coleoptera (Scolytidae) | Real-time PCR of COI | Propylene glycol | Lindgren funnel trap with PG | 95% ethanol | n.a. | well preserved for morphological and molecular analyses |
| Wiesman et al. (2016)          | 2016    | Coleoptera (Carabidae)  | Sanger sequencing of COI, ITS2, 18S and 28S | Propylene glycol | pitfall trap with PG | n.a. | pinned and dried | n.a. | well preserved for morphological analysis |
| Boo et al. (2017)              | 2017    | Diptera (Tephritidae)  | Sanger sequencing of COI and microsatellite genotyping | Propylene glycol | specimens directly placed in pure PG | 95% ethanol (−20 °C) | n.a. | well preserved for morphological and molecular analyses |
| Boo et al. (2017a), Boo et al. (2017b), Boo et al. (2017) | 2017    | Diptera (Tephritidae)  | Sanger sequencing of COI and microsatellite genotyping | Propylene glycol | specimens directly placed in pure PG | 95% ethanol (−20 °C) | n.a. | effective shipping of samples; well preserved for morphological and molecular analyses |
| Bowers et al. (2017)           | 2017    | Arthropod sweep net bulk sample | COI metabarocoding | Propylene glycol antifreeze (Uni-Gard-100) | specimens directly placed in pure PG | 100% ethanol, rinsed with PBS prior to extraction | 21 days | effective shipping of samples; well preserved for morphological and genomic analyses |
| Cordaro et al. (2017)          | 2017    | aquatic insects | Sanger sequencing of COI | Propylene glycol | specimens directly placed in 80% PG | n.a. | 95% ethanol | n.a. | specimens suitable for barcoding |
| Greenslade et al. (2017)       | 2017    | Collembola (Dicyrtomidae) | Sanger sequencing of COI | Mono-propylene glycol (antifreeze) | pitfall trap with PG and soap | Noctilucent solution over night | well preserved for morphological and molecular analyses |
| Hoekman et al. (2017)          | 2017    | Coleoptera (Carabidae)  | Sanger sequencing of COI | Propylene glycol | pitfall trap with 50% PG | 95% ethanol, renewed after 24 h | n.a. | specimens highly suitable for morphological and genetic identification |

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DNA suitable for population specimens well preserved for Vane trap effective transport of samples; Preservation step pitfall trap with n.a. Condition(s) well preserved for morphological 70% ethanol Duration 1, 6 and 12 Arachnida (Acari) well preserved for molecular specimens well preserved for morphological n.a. n.a. n.a. Hemiptera Arachnida Diptera Central outcomes Hymenoptera Coleoptera Lepidoptera Lepidoptera insect (pH 2) Propylene glycol Pan trap with PG n.a. 70% ethanol few months (10%) well preserved for morphological and molecular analyses

Gregoire Taillefer and Wheeler (2018) 2018 Arthropod sweep net bulk sample DNA suitable for population specimens well preserved for morphological 50% PG and soap pan trap with 50% PG and soap 7–8 days 95% ethanol or air-dried (RT) n.a. well preserved for morphological and molecular analyses

Ide et al. (2018) 2018 Hymenoptera (Formicidae) DNA suitable for population specimens well preserved for morphological Propylene glycol baited pan trap with pure PG 3 h washed in 95.5% ethanol, air-dried (RT) n.a. well preserved for molecular analysis

Matari et al. (2018) 2018 Diptera (Calicidae; gut content) DNA suitable for population specimens well preserved for morphological Propylene glycol suction trap with 50% PG weekly from May to October 95.5% ethanol (−20 °C) n.a. specimens well preserved for barcoding and microbial gut content analysis

Angelella et al. (2019) 2019 Hymenoptera (Apidae) DNA suitable for molecular analysis Propylene glycol pitfall trap with 25% PG weekly for 14 weeks undiluted ethanol (−80 °C) n.a. DNA suitable for population genomic SNP analysis

Bowser et al. (2019) 2019 Arthropod sweep net bulk sample DNA suitable for population specimens well preserved for morphological Propylene glycol antifreeze (Uni-Gard -100) specimens directly placed in pure PG n.a. (−25 °C) 100% ethanol, rinsed with PBS prior to extraction 1 week well preserved for genomic analysis

DiGirolomo et al. (2019) 2019 Coleoptera (Buprestidae) DNA suitable for population specimens well preserved for morphological Propylene glycol barrel with baited collection cups with PG several months n.a. n.a. specimens well preserved for morphological and molecular analyses

Jusino et al. (2019) 2019 Arthropod pitfall trap bulk sample DNA suitable for population specimens well preserved for molecular Propylene glycol pitfall trap with 25% PG 2–3 days 100% ethanol (RT, −20 °C) n.a. well preserved for morphological and genomic analyses

Krosh et al. (2019) 2019 Diptera (Tephritidae) DNA suitable for population specimens well preserved for molecular Propylene glycol specimens directly placed in pure PG n.a. (RT) effective shipping of samples; well preserved for molecular and genomic analyses

Landi et al. (2019) 2019 Coleoptera (Curculionidae) DNA suitable for population specimens well preserved for molecular Propylene glycol ethanold-baited Lindgren funnel trap with PG n.a. (Nov-Feb) n.a. n.a. well preserved for morphological and molecular analyses

Lenthard and Schaffer (2019) 2019 Arachnida (Acari) DNA suitable for population specimens well preserved for genomic PCR-based visualisation Propylene glycol exposure to ethyl acetate vapour, followed by dehydration in 95.5% ethanol 30 °C and dehydration in 95.5% ethanol both 24 h 99% PG 1, 6 and 12 months all replicates with PG-preserved (n = 12) were successfully amplified for all timepoints and three different fragment sizes

Nakahama et al. (2019) 2019 Orthoptera (Gryllidae) DNA suitable for population specimens well preserved for molecular PCR-based visualisation (COI) Propylene glycol pitfall trap with PG ~30 days 95% ethanol for transport; 100% ethanol storage n.a. well preserved for morphological and molecular analyses, although heavy rainfall diluted PG in traps and has led to sediment wash-in

Ramirez et al. (2019) 2019 Arachnida (Araneae) DNA suitable for population specimens well preserved for molecular Sanger sequencing (COI, 12S, 16S, H3, 18S and 28S) Propylene glycol (Sierra antifreeze) pitfall trap with PG ~30 days 95% ethanol for transport; 100% ethanol storage n.a. well preserved for morphological and molecular analyses

Taillefer and Wheeler (2019) 2019 Diptera (diverse Schizophora families) DNA suitable for population specimens well preserved for molecular Sanger sequencing (COI) Propylene glycol pitfall trap with PG, drop of detergent 6–8 days 95% ethanol n.a. well preserved for morphological and molecular analyses

Ballare et al. (2020) 2020 Hymenoptera (Anthophila) DNA suitable for population specimens well preserved for molecular ddRAD sequencing Propylene glycol Vane trap with PG, amongst other treatments (e.g. pan trapping with soapy water; netted specimens placed in ethanol) 5 days 100% ethanol n.a. (until pinning) all treatments produced a large number of high-quality loci (~4,000, ~20×). In comparison, the two PG-preserved species showed average DNA concentrations, but higher than average mean locus depths and lower than average mean numbers of polymorphic loci

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not to be underestimated amount of water and other compounds (Nagy 2010; Goetze and Junghbluth 2013; Borges et al. 2016), but chemical interactions and their effects on medium- to long-term DNA integrity are largely unknown. In addition to high temperatures, water dilution of PG in principle leads to a generally lower DNA integrity (Stevens et al. 2011; Höfer et al. 2015; Patrick et al. 2016; Nakamura et al. 2020), since water can lead to the hydrolysis of nucleic acids. Since most reviewed studies did not indicate the nature of the propylene glycol used, or field and storage temperatures, it was difficult to compare study outcomes. Nevertheless, in several studies, PG was used in the range of 20–80% as a trapping agent and traps deployed for a duration of 1–2 weeks. Specimens collected and fixed under these conditions were still sufficiently preserved for DNA analysis (Coulson et al. 2005; Sonoda et al. 2010; Hoekman et al. 2017; Angelella et al. 2019). In one of the most extreme cases, samples were fixed for 1 month in pitfall traps containing PG-antifreeze. The traps further experienced heavy rainfall and sediment wash-in. Nevertheless, Ramirez et al. (2019) successfully amplified six marker genes for the spider family under investigation and were able to morphologically investigate the specimens at hand.

Propylene glycol is in accordance with the Dangerous Goods Regulations of The International Air Transport Association (IATA). This means that PG-fixed samples are suitable for direct shipping and do not have to be transferred to another chemical agent on the spot. This characteristic was especially important for studies in remote areas (e.g. Schütze et al. 2012; Haase and Zielske 2015; Patrick et al. 2016; Bagnall 2016; Boontop et al. 2017a). In addition, it was seen as advantageous that PG can be relatively easily and cheaply obtained as an antifreeze in many parts of the world – a fact that does not always apply to absolute ethanol. As a major drawback, however, PG-based antifreezes might be regarded as special waste. As such, it can be prohibited to introduce them into local septic systems and the natural ground or to dispose them as domestic waste (Thomas 2008; Renaud 2012). Some national programmes might even prohibit the general use of antifreezes, as they can come along with additives such as lubricants, buffers, corrosion inhibitors and anti-foaming agents, whose impacts on natural environments are often not totally understood – or are considered carcinogenic (e.g. Tolytriazole, see Thomas 2008). Yet, much of the concern and regional bans relate to ethylene glycol-based antifreezes, which are toxic to humans and impose environmental risks. Readily available food-grade PG-based formulations such as from swimming pool or recreational vehicle antifreezes are generally regarded as safe (GRAS) material. They metabolize to lactic acid or substances of the Krebs cycle, which are natural metabolite products in the environment (Thomas 2008; Skvarla et al. 2014). Nevertheless, the waste-disposal issue imposed by using antifreeze fixatives (as it is true for larger quantities of ethanol as well) should be understood beforehand and appropriate measures taken.

Another relevant aspect refers to a potential catch bias caused by the fixative. Although Schmidt et al. (2006) have not integrated PG in their test, they showed that capture efficiencies of commonly used fixatives in pitfall trapping of spiders and carabids can greatly vary. Adding to this, Weeks Jr & McIntyre (1997), Calixto et al. (2007) and McCravy and Willand (2007) demonstrated that PG might not only affect the size of the sample but also its taxonomic composition. Höfer et al. (2015) reported that pitfall traps deployed with PG captured significantly more spider species, but were not selectively attractive for par-
Application of propylene glycol as a preservative

Propylene glycol was only occasionally used as a medium-term preservative or storage medium. After PG fixation, most samples were stored in ≥95% ethanol until DNA extraction or PG-fixed samples directly analysed within a few days when retrieved from the field. Nevertheless, Nakamura et al. (2020) highlighted the potential use of PG as a chemical agent that can be applied from trapping to storage and for various taxonomic groups, so that hands-on times can be shortened as well as labour and equipment costs reduced (i.e. no specimen picking, circumventing transfer into another solution and container). The authors compared the COI-amplification rates of dipteran, hymenopteran and coleopteran specimens preserved in 98% PG and 99.5% ethanol over a period of 2 weeks to >6 months at room temperature, concluding that DNA might be more long-term stable in PG than in ethanol. On the contrary, Patrick et al. (2016) tested various storage agents (including 99.5% PG and 97–100% ethanol) applicable in remote areas on three dipteran species under different temperatures. Although the experiment ended after 15 days, their results suggest that keeping PG fixed samples as cold as possible (e.g. packed in ice-filled boxes from hotel bars) is important to ensure short- to mid-term DNA integrity.

If we adopt the results to common practices of sample storage, it tells us that PG-preserved samples should preferably be stored cool and dark just like ethanol-preserved samples. Yet, DNA quality and quantity of long-term stored, chilled PG-preserved samples should be investigated in further detail.

Application of propylene glycol in HTS studies

Sufficiently high DNA quantities and DNA qualities are prerequisites in HTS studies. Lienhard and Schäffer (2019) evaluated DNA quality and quantity of ethanol- and PG-preserved oribatid mite species (<1 mm, preserved for several weeks), originating from seven DNA isolation methods suitable for high-throughput DNA sequencing. Although some study parameters had a significant effect on DNA quantity and quality, results for specimens preserved in PG or absolute ethanol generally suggest a high comparability. Similarly, Carter (2003) investigated the molecular weight spectra of double-stranded DNA (dsDNA) for specimens of the Rough Woodlouse Porcellio scaber preserved in PG, ethanol, ethyl acetate and 2-ethoxy ethanol for 12 months at room temperature. Ethanol- and cryo-preserved specimens provided the best quality and highest concentration of high molecular weight dsDNA for the investigated time period (with a remarkable drop after 24 months for ethanol). Yet, DNA quality (in terms of degradation) and DNA quantity (in terms of concentration) of specimens stored in PG were decreased, especially for longer fragments (>5 kbp).

However, specimens were stored at room temperature, which showed a strong degradation effect in Patrick et al. (2016) compared to samples which were kept cool.

Systematic studies analysing the impact of PG fixation and preservation in the context of HTS are widely lacking. Still, first case studies indicate a high applicability of PG fixation for short read amplicon sequencing (metabarcoding; Bowser et al. 2017, 2019; Lefort et al. 2017; Jusino et al. 2019; Liu et al. 2020; Robinson et al. 2020). In particular Robinson et al. (2020) tested the effect of PG antifreeze fixation of homogenised mock communities and benthic bulk samples. Their COI metabarcoding results indicate a generally high comparability of communities from ethanol and PG fixed samples. The latter even produced a higher proportion of arthropod reads and a higher richness of exact sequence variants (ESVs) when compared to ethanol samples.

Besides studies on short read amplicon sequencing, the applicability of PG was shown for short read reduced representation methods (RAD-sequencing; Perry et al. 2017; Angelella et al. 2019; Ballare et al. 2020). In particular, Ballare et al. (2020) conducted a comparative setting testing the impact of different field sample methods for SNP detection in wild bees, including PG-filled Vane traps. While a suitable DNA concentration and high locus depth were found for PG-fixed specimens, the number of loci recovered was comparatively low. Still, the two targeted wild bee species on average possessed more than 10,000 loci with a mean locus depth of >70. One potential cause for the lower than average mean number of polymorphic loci might be an incomplete PG evaporation, leading to an insufficient ethanol preservation prior to pinning. Alternatively, PG-stored samples were kept at room temperature, whereas other samples which showed higher numbers of polymorphic loci were stored at -20 °C.

To the best of our knowledge, no study used PG-fixed or -preserved specimens for invertebrate genome sequencing. Our assumption, however, would be that if samples are fixed and stored under optimal conditions, genome sequencing based on short read lengths should be possible. However, how ultra-long sequencing (e.g. Nanopore) will be affected by potential DNA degradation effects of PG remains unclear and should be explicitly addressed.

Integrative use cases of propylene glycol-conserved invertebrate samples

Besides the possibility to perform DNA-based analyses (e.g. microsatellite fingerprinting, DNA barcoding, metabarcoding and RAD-seq) directly on the invertebrates trapped or stored in PG, the samples seem suitable for a variety of research designs. Firstly, it has to be highlighted that PG-conserved specimens demonstrate a reduced shrinkage effect and specimens often remain appropriately conserved for morphological examina-
Conclusions

There is currently only limited scientific literature on the use of PG for DNA-based analyses of invertebrates available, and even less so in the context of HTS. However, the investigated studies indicate that PG can be a versatile and worthwhile alternative for sample fixation (and potentially preservation) of various organism groups and in a range of methodological setups. Yet, generally valid statements about fixatives and preservatives are difficult to make (Nagy 2010; Short et al. 2018), and can be biased by comparing agents with contrasting water conditions affecting DNA integrity (see Nakamura et al. 2020), varying field vs. laboratory humidity conditions (due to the hygroscopic nature of PG) and storage temperatures or by confusing chemicals (e.g. Vaudo et al. 2018).

Future studies which plan the application of PG should critically scrutinize their trapping, specimen and storage conditions. For how many days are traps deployed? How will humidity, precipitation, UV exposure and temperature conditions in the sampling area affect the fixative? Do the targeted organisms allow for an easy tissue penetration by the fixative (e.g. soft-bodied vs. sclerotinised specimens)? Can PG reduce hands-on times and yet overall costs (e.g. no sample transfer for shipping; no evaporation prior to DNA isolation) (see e.g. Robinson et al. 2020)? In many cases, and in particular for new large-scale DNA-based monitoring programmes, one should not simply go by tradition but perform environment- and target group-specific tests and cost calculations before deciding upon the most suitable fixative.

Acknowledgements

The work is part of the EU COST Action CA15219 (“DNAqua-Net”). It was financially supported by the German Research Foundation (grant WE 6055/1-1) and the Bauer and Stemmmer foundations program “FORSCHUNGSGEIST! Next Generation Sequencing in der Ökosystemforschung”.

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Supplementary material 1

Overview of DNA-based studies of invertebrates applying propylene glycol, sorted by year and taxonomic group

Author: Alexander Weigand

Data type: study counts

Explanation note: Overview of DNA-based studies of invertebrates applying propylene glycol, sorted by year and taxonomic group.

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