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Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter

Johan Spens[1,2]†, Alice R. Evans[1]†, David Halfmaerten[3], Steen W. Knudsen[1], Mita E. Sengupta[4], Sarah S. T. Mak[1], Eva E. Sigsgaard[1] and Micaela Hellström[1,5]*

[1]*Centre for GeoGenetics, Natural History Museum of Denmark, Øster Voldgade 5-7, 1350 Copenhagen K, Denmark;* [2]*Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Skogsmarksgränd, 90183 Umeå, Sweden;* [3]*Research Institute for Nature and Forest, Gaverstraat 4, 9500 Geraardsbergen, Belgium;* [4]*Department of Veterinary Disease Biology Parasitology and Aquatic Diseases, Dyrlægevej 100, 1870 Frederiksberg C, Copenhagen, Denmark; and* [5]*Department of Ecology, Environment and Plant Sciences, Stockholm University, 10691 Stockholm, Sweden*

Summary

1. Aqueous environmental DNA (eDNA) is an emerging efficient non-invasive tool for species inventory studies. To maximize performance of downstream quantitative PCR (qPCR) and next-generation sequencing (NGS) applications, quality and quantity of the starting material is crucial, calling for optimized capture, storage and extraction techniques of eDNA. Previous comparative studies for eDNA capture/storage have tested precipitation and 'open' filters. However, practical 'enclosed' filters which reduce unnecessary handling have not been included. Here, we fill this gap by comparing a filter capsule (Sterivex-GP polyethersulfone, pore size 0·22 μm, hereafter called SX) with commonly used methods.

2. Our experimental set-up, covering altogether 41 treatments combining capture by precipitation or filtration with different preservation techniques and storage times, sampled one single lake (and a fish-free control pond). We selected documented capture methods that have successfully targeted a wide range of fauna. The eDNA was extracted using an optimized protocol modified from the DNeasy® Blood & Tissue kit (Qiagen). We measured total eDNA concentrations and Cq-values (cycles used for DNA quantification by qPCR) to target specific mtDNA cytochrome *b* (cyt *b*) sequences in two local keystone fish species.

3. SX yielded higher amounts of total eDNA along with lower Cq-values than polycarbonate track-etched filters (PCTE), glass fibre filters (GF) or ethanol precipitation (EP). SX also generated lower Cq-values than cellulose nitrate filters (CN) for one of the target species. DNA integrity of SX samples did not decrease significantly after 2 weeks of storage in contrast to GF and PCTE. Adding preservative before storage improved SX results.

4. In conclusion, we recommend SX filters (originally designed for filtering micro-organisms) as an efficient capture method for sampling macrobial eDNA. Ethanol or Longmire's buffer preservation of SX immediately after filtration is recommended. Preserved SX capsules may be stored at room temperature for at least 2 weeks without significant degradation. Reduced handling and less exposure to outside stress compared with other filters may contribute to better eDNA results. SX capsules are easily transported and enable eDNA sampling in remote and harsh field conditions as samples can be filtered/preserved on site.

Key-words: capsule, eDNA capture, environmental DNA, extraction, filter, monitoring, quantitative PCR, species-specific detection, water sampling method

Introduction

The realization that DNA from macrobiota can be obtained from environmental samples (environmental DNA, eDNA) started with excrements (Höss *et al.* 1992) and sediments (Willerslev *et al.* 2003). Over the last decade, the potential of aqueous eDNA to identify a wide range of plants and animals from a small volume of water has been realized (Martellini, Payment & Villemur 2005; Thomsen *et al.* 2012; Rees *et al.* 2014). Aqueous eDNA is an emerging increasingly sensitive technique for revealing species distributions (e.g. Jane *et al.* 2015; Valentini *et al.* 2016), early detection of invasive species (e.g. Smart *et al.* 2015; Simmons *et al.* 2016) and monitoring rare and/or threatened species for conservation (e.g. Zhan *et al.* 2013; McKee *et al.* 2015). Aqueous eDNA monitoring provides possibilities to upscale species distribution surveys considerably, because much less effort in time and resources are required compared to conventional methods (Dejean *et al.* 2012; Davy, Kidd & Wilson 2015). Based on literature

*Correspondence author. E-mail: micaela.hellstrom@su.se
†Joint first authors.

searches, we catalogue 49 studies successfully applying eDNA from water samples to detect macro-organisms in aquatic ecosystems, published between January 2005 and March 2015 (when this study was initiated; Table S1, Supporting Information). To our knowledge, 39 additional empirical studies were published since then, indicating a rapid rise of interest in this research area (Table S2).

The field of eDNA is still evolving, and a consensus of capture, storage and extraction methods has not yet been reached (Goldberg, Strickler & Pilliod 2015; Tables S1 and S2). In fact, the diversity of methods is almost as high as the number of research groups investigating this fairly new field of research. To ensure reliable results of downstream applications such as quantitative PCR (qPCR) and next-generation sequencing (NGS), the quantity and quality of the starting material is crucial. From our eDNA laboratory experience, we find that a modified easy-to-follow extraction protocol resulting in high yields is needed. Based on eDNA studies published so far (Tables S1 and S2), we identify three pre-PCR key issues that hold opportunities for improvement: (i) capturing sufficient quantities of eDNA as quite a few studies report low amounts of captured total eDNA, (ii) effectively preserving eDNA samples before extraction and (iii) lowering contamination risks from collection to extraction of eDNA.

Comparative studies on aqueous eDNA capture and storage techniques (i.e. optimal ways of preserving the eDNA captured on the filters until extraction; e.g. Renshaw *et al.* 2015) were based on the so-called 'open filters' (requiring handling, a filter funnel and a vacuum pump; e.g. Liang & Keeley 2013; Turner *et al.* 2014b) and ethanol precipitation (EP; e.g. Piaggio *et al.* 2014; Deiner *et al.* 2015). However, no enclosed filters were included in previous comparative assays.

The Sterivex-GP capsule filter (SX), with a polyethersulfone membrane, is a standard method for characterizing microbial communities (Chestnut *et al.* 2014) and for removing pathogens from water as the organisms are captured on the filter membranes. To our knowledge, only two published aqueous eDNA studies have used this filter to detect aquatic macro-organisms (fish detection: Keskin 2014; Bergman *et al.* 2016), and the technique has been successful to detect a wide range of aquatic macro-organisms in Denmark and Belgium (M. Hellström, M.E. Sengupta, S.W. Knudsen, D. Halfmarten. unpublished, S1). The SX filter is enclosed in a capsule, which reduces handling. A water sample can easily be filtered in the field, saving time and facilitating fixation of the eDNA immediately after capture. Additionally, downstream DNA extraction takes place within the filter capsules with no need for the membrane to be removed or handled. We therefore test the performance of SX compared to other more frequently used eDNA capture methods (Table S1), under different storage conditions, in an effort to address issues 1–3 above. To date, there are no studies comparing SX to other capture methods and multiple storage treatments. We aim to fill this gap, with an experimental study comparing SX with four other capture methods in a set-up with five typical storage treatments and three different storage times (up to 2 weeks). The tested open filter materials polycarbonate, cellulose nitrate and glass fibre (GF) and the range of tested pore sizes (0·2–0·6 μm) are typical of previous studies (Tables S1 and S2). We used an optimized extraction protocol based on a commercial kit to increase eDNA yields. To evaluate the usefulness of the SX and preservation buffers in comparison with typically used methods (Tables S1 and S2), we test the following H_0 hypotheses:

$H_0$1. CAPTURE METHOD: SX is equally effective as other tested eDNA capturing techniques in regard to DNA quantity and quality measured as the total extracted eDNA concentration [$eDNA_{tot}$] and as Cq-values (quantification cycles, *sensu* Bustin *et al.* 2009) from two species-specific qPCR assays.

$H_0$2a. STORAGE PRESERVATIVE: Storing filters with a preservation buffer does not affect qPCR amplification compared to immediate extraction or freezing at −20 °C (no buffer added).

$H_0$2b. STORAGE TIME: There is no significant difference in eDNA quality over time between SX and the other tested capturing techniques.

$H_0$3. CONTAMINATION: There is no significant difference between SX and the other tested capture techniques in occurence of false positives.

To test these hypotheses, we use an experimental set-up with subsampling a single large homogenous sample of water from a Danish lake. Subsamples are subjected to different eDNA capture methods within the same day followed by different storage treatments. A control site (fish-free pond) is sampled using the same set-up. Each capture and storage treatment is assessed using concentration of total eDNA as well as species-specific qPCR assays targeting pike *Esox lucius* L. and perch *Perca fluviatilis* L. By testing H_0 hypotheses (1–3), the multiple opportunities for optimization of eDNA surveys held by the use of SX may be empirically evaluated. Based on the results, we suggest recommendations for improved capture, storage and extraction to use for aqueous eDNA, taking remote and harsh field conditions into consideration.

Materials and methods

STUDY SITES

We chose Gentofte Lake, Denmark (N55·7435°, E12·5348°), as the study site and a fish-free pond in Copenhagen botanical garden as a negative field control (N55·6875°, E12·5746°). Gentofte Lake (26 ha) is an alkaline clear water (Appendix S2) harbouring a wide range of fish species, including pike and perch.

WATER COLLECTION

We retrieved 130 L of water from Gentofte Lake on 17 March 2015. The water (4 °C) was collected at *c.* 30 points along *c.* 100 m of shoreline close to the outlet of the lake. Additionally, we collected 40 L of water from the control pond on 21 March 2015. The water was

collected in sterilized 5-L buckets which prior to sampling were soaked in bleach (5%) for 10 min, and then rinsed with laboratory-grade ethanol (70%). The containers were soaked repeatedly in lake water at a location away from the collection point. Nitrile gloves were used during cleaning, collection and filtration.

CAPTURE AND STORAGE

We carried out 41 different treatment combinations of the water sample in total (Table 1, Fig. S1). We used five capture techniques, five storage methods and three time regimes. All treatments were performed in triplicate. Apart from an in-house modified SX procedure (see Fig. 1), the capture and storage methods were based on published sources (Table S1). The capture methods (hereafter referred to with their abbreviations in square brackets) were as follows: (i) ethanol precipitation [EP] (Ficetola *et al.* 2008), (ii) mixed cellulose esters membrane filters including cellulose nitrate and cellulose acetate [CN]; Advantec 47 mm diameter 0·45 μm pore size (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), (iii) polycarbonate track-etched filters [PCTE]; Whatman Nucleopore Membrane 47 mm diameter 0·2 μm pore size (Merck KGaA, Darmstadt, Germany)], (iv) glass fibre [GF] membrane filters; Advantec GA-55 47 mm diameter 0·6 μm pore size (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and (v) sterivex-GP capsule filters [SX]; polyethersulfone 0·22 μm pore size with luer-lock outlet (Merck KGaA)]. Further downstream, SX was divided into an extraction from the filter within the capsule ($SX_{CAPSULE}$), after removal of the storage buffer, and an extraction from the removed preservation buffer within a centrifuge tube (SX_{TUBE}; see DNA extraction section below). The different storage methods were as follows: (i) ethanol 99% 200 proof at room temperature (RT), Molecular Biology Grade (Thermo Fisher Scientific Inc., Waltham, MA, USA); (ii) Longmire's buffer at RT (Longmire's; Longmire, Maltbie & Baker 1997); (iii) RNAlater at RT (RNA Stabilization Reagent; QIAGEN, Stockach, Germany); (iv) no buffer, frozen at −20 °C; and (v) no buffer, refrigerated at 8–10 °C. The three time regimes between filtration and extractions were (i) within 5 hours (5 h), (ii) within 24 h and (iii) after 2 weeks. Each treatment (n = 41) was performed in triplicate. For each filter replicate, 1 L of lake water was processed (0·015 L for EP). For each capture–storage treatment, we included one negative control without lake water. Additionally, 1 L tap water was run through each filter (0·015 L for EP) as a control to detect potential contamination from the filtration facilities. For the control pond, one sample per capture–storage treatment was processed (n = 23). We captured eDNA from 155 subsamples and negative controls altogether. The water samples were filtered or ethanol-precipitated by a team of 10 researchers and the replicates of each treatment started at different times to avoid temporal bias of filtrations. Prior to DNA capture, bench surfaces and all equipment were wiped with bleach (5%) and laboratory-grade ethanol (70%). Prior to each collection of subsamples, the water was mixed thoroughly in the 130-L container. For the open membrane filter (GF, CN and PCTE), 1 L water samples were vacuum-filtered (*c.* 15–30 min) using Nalgene 250-mL sterile disposable test filter funnels (Thermo Fisher Scientific Inc. USA). The filters were removed from the funnel with forceps and then placed in 5-mL DNA LoBind® centrifuge tubes (Eppendorf AG, Hamburg, Germany) that were either empty (if the time regime was 5 h or the storage method was freezing) or contained preservation buffer. For all treatments and downstream applications, Eppendorf DNA LoBind® tubes were used in order to avoid up to 50% retention of DNA by the plastic, which is a documented problem especially for short DNA fragments (Gaillard & Strauss 1998; Ellison *et al.* 2006). For the SX filters, 1 L of water was slowly (*c.* 10 min to avoid tearing of filters, following manufacturer's recommendations) pushed through each filter capsule using a prepacked sterile 50-mL luer-lock syringe. Remaining water in the SX was removed by pushing air through the filter until dry, also using the syringe. The outlet ends of the filters were closed with MoBio outlet caps (MOBIO Laboratories, QIAGEN) and 2 mL preservation buffer was pipetted to the inlet end using filter tips. The inlet ends were closed with inlet caps (MOBIO Laboratories, QIAGEN) and both ends were sealed with parafilm whereafter the capsules were inverted vigorously. The frozen samples and the (5 h) and (24 h) EP samples were placed at −20 °C until extraction, while the non-treated samples (5 h) were placed in a refrigerator and extracted directly after the filtering session. Samples containing buffers were stored at RT until processed. The (2 weeks) EP samples were frozen for 24 h prior to extraction to allow for precipitation. In total, we processed 96·135 L of water from the lake (32 treatments × 3 replicates × 1 L + 3 EP treatments × 3 replicates × 0·015 L) and 20·045 L of water from the control pond (20 treatments × 1 replicate × 1 L + 3 EP treatments × 1 replicates × 0·015 L; Table 1).

MOLECULAR LABORATORY CONDITIONS

DNA extractions and qPCR assays took place in the laboratories at the Centre for GeoGenetics, University of Copenhagen, Denmark. The facilities are designed for handling environmental samples requiring the most stringent precautions to avoid contamination. Pre-PCR, extraction and PCR facilities are located in separate designated rooms with positive air pressure. Laboratory coats are changed between rooms. Prior to any work in the laboratory, all surfaces are washed with 5% bleach and 70% ethanol. After completing extractions

Table 1. Outline of the number of samples processed per capture and storage treatment (negative control pond in parentheses)

		Storage								
			Frozen	Ethanol	Longmire's	RNAlater	Frozen	Ethanol	Longmire's	RNAlater
Capture	Sum	Refrigerated 5 h	24 h				2 weeks			
$SX_{CAPSULE}$	27 (5)	3 (1)	3 (1)	3 (1)	3 (1)	3 (1)	3	3	3	3
SX_{TUBE}	18 (3)			3 (1)	3 (1)	3 (1)		3	3	3
Cellulose nitrate	15 (5)	3 (1)	(1)	(1)	(1)	(1)	3	3	3	3
Glass fibre	27 (5)	3 (1)	3 (1)	3 (1)	3 (1)	3 (1)	3	3	3	3
Polycarbonate	27 (5)	3 (1)	3 (1)	3 (1)	3 (1)	3 (1)	3	3	3	3
Precipitation	9 (3)	3		3 (3)				3		
Total	123 (26)									

Sterivex, eDNA extraction within capsule ($SX_{CAPSULE}$); Sterivex, eDNA extraction from buffer in tube outside capsule (SX_{TUBE}).

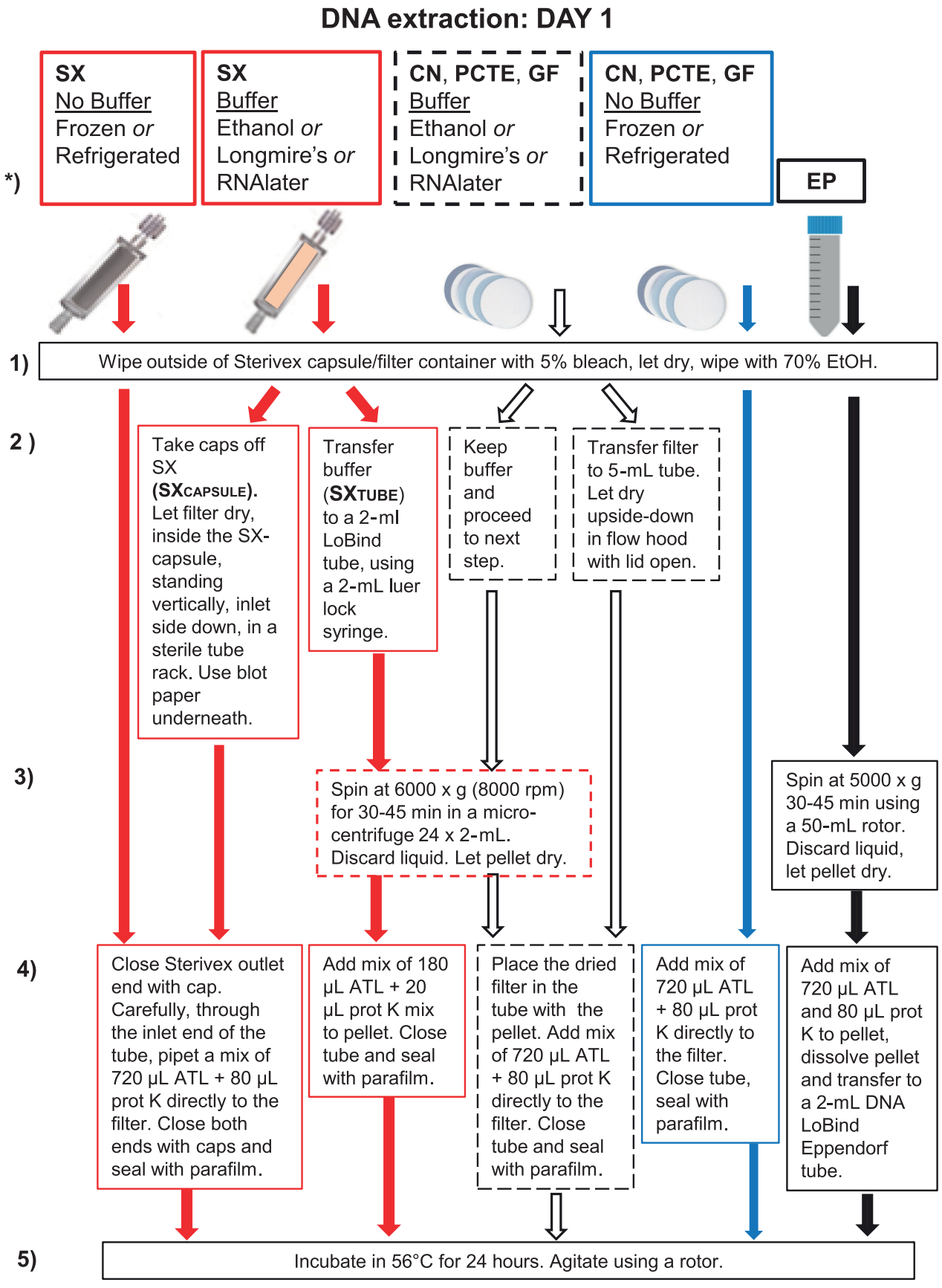

Fig. 1.

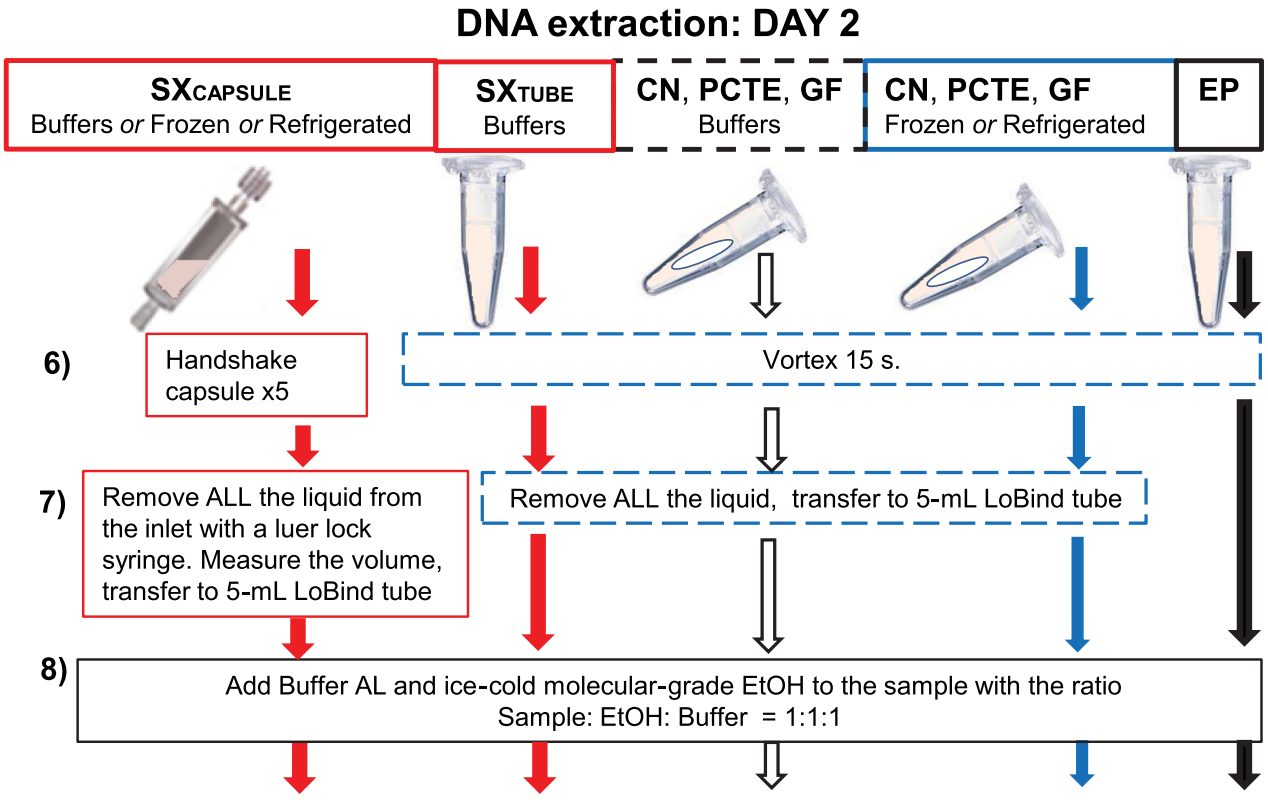

Fig. 1. Flow chart illustrating the modified environmental DNA (eDNA) extraction protocol based on DNeasy Blood & Tissue Kit (QIAGEN, Carlsbad, CA, USA). *) Capture: SX, Sterivex-GP polyethersulfone capsule filters, Note that $SX_{CAPSULE}$ and SX_{TUBE} are treated as separate samples from step 2. CN, cellulose nitrate; PCTE, polycarbonate track-etched; GF, glass fibre filters; EP, ethanol precipitation. Storage: Frozen at −20 °C, Refrigerated are samples stored at 8–10 °C and processed within 5 h. Steps 9–26 see Appendix S1.

involving guanidiumthiocyanate, surfaces are washed with 70% ethanol (to avoid reactions between chlorine in the bleach and guanidiumthiocyanate in two of the buffers provided with the Qiagen kit), 5% bleach and then 70% ethanol. All extractions of eDNA took place in laminar flow hoods which were UV-treated before and after extractions. Every night, the entire facilities are automatically UV-treated for a 2-h period.

DNA EXTRACTION

We extracted the eDNA using the extraction protocol outlined in Fig. 1 and Appendix S1. The SX filters containing preservation buffers underwent two extractions, one extraction from the buffer and one extraction within the filter capsule after it had been emptied of buffer (hereafter referred to as SX_{TUBE} and $SX_{CAPSULE}$). Altogether, 179 (24 SX_{TUBE} + 155 (see 'Capture and storage' section above) samples from the study lake and the control pond were extracted. We measured [$eDNA_{tot}$] in each extraction using a Qubit 1.0 fluorometer (Thermo Fisher Scientific Inc.) applying the high-sensitivity assay for dsDNA (Life Technologies, Carlsbad, CA, USA).

QUANTITATIVE PCR

For the qPCR assays (e.g. Wilcox *et al.* 2013), two species-specific TaqMan primers/probe sets were used targeting 84 and 89 base pair fragments of the mitochondrial cytochrome *b* (cyt *b*) gene in pike and perch, respectively (Table S3). Species specificity of the assays was tested on extracted DNA from non-target species (Table S3) using the qPCR set-up described below. These non-target species did not generate any amplification signals. The optimal ratio of probe: primer concentration was tested prior to the study. The final PCR set-up to detect the target species was as follows: pike – 5 μL template DNA, 12·5 μL TaqMan Environmental Master Mix 2·0 (Life Technologies), 3 μL forward primer (10 μM), 2 μL reverse primer (10 μM) and 3 μL probe (2·5 μM); and perch – 5 μL template DNA, 12·5 μL TaqMan Environmental Master Mix 2·0 (Life Technologies), 0·5 μL forward primer (10 μM), 2·5 μL reverse primer (10 μM), 3 μL probe (2·5 μM) and 1·5 μL UV-treated laboratory-grade water. The TaqMan qPCRs were performed on a Stratagene Mx3005P (Thermo Fisher Scientific Inc.) using thermal cycling parameters of 50 °C (5 min), 95 °C (10 min) followed by 50 cycles of 95 °C (30 s) and 60 °C (1 min). For each plate, no-template controls (NTCs) and positive/negative tissue extracts were run alongside the samples. All filtering and extraction negatives were included in the qPCR assays. Additional qPCR replicates were run in order to detect effects of freezing and thawing of the samples. To check for PCR inhibition in the lake, separate qPCR assays for both species following the protocols above were performed in a dilution series (1 : 1, 1 : 2, 1 : 10 and 1 : 20) of extracted DNA on four samples replicated twice plus two positive and two negative controls to determine any deviation of the amplification curves. The dilution series did not indicate inhibition.

DATA ANALYSIS

To compare detection probability (i.e. diagnostic sensitivity) between eDNA capture methods, the proportion of positive qPCR replicates was calculated for each target species. Positive samples were analysed

using multivariate decision trees and univariate tests of 'no-effect' null hypotheses. To explore the effect of capture and storage on qPCR Cq-values, Chi-square Automatic Interaction Detector (CHAID) decision tree was used. CHAID is a nonparametric tree-building method that can handle multivariate categorically induced quantitative responses (IBM Corp. (2013)). It defines optimal multiway splits and adjusts for Bonferroni. The main advantage of this approach is to analyse a data set all-in-one (rather than manually splitting the data into user-selected subgroups and thereafter choosing and performing multiple tests). The approach offers a number of other advantages including its ability to handle categorical (ordered, nominal) data types well and to model nonlinear relationships without having to specify *a priori* the form of the interactions. A CHAID tree produces an overview, grouping or singling out the factors that predict the variation in the response variable. Categorical variables (capture method, storage treatment and storage time) were used as model predictors, and Cq-value from qPCR was set as the response target. Two trees were generated: the first targeting perch and the second pike. Tree depth, that is the maximum number of branching levels, was set to two (realized from ten 50/50 split validations) to reduce overfitting.

For a univariate test of H_0 (1–2a,b), first a Wilcoxon signed-rank test for paired samples was applied to determine whether [$eDNA_{tot}$] and Cq-values attained using $SX_{CAPSULE}$ differ significantly, from any of the other tested capture methods (CN, GF, PCTE, EP and SX_{TUBE}). Secondly, SX, GF and PCTE filter results were tested for signs of eDNA degradation over time, that is detecting any significant difference in Cq-values or [$eDNA_{tot}$] between 24 h and 2 weeks of storage. Wilcoxon signed-rank test was used as data exhibited non-normal distributions. Thirdly, guided by results from the CHAID trees, results from $SX_{CAPSULE}$ stored in ethanol or Longmire's were tested (Mann–Whitney) for differences in Cq-value against $SX_{CAPSULE}$ without preservation buffer. The CN filter group was reduced, as the planned 1-day storage treatment was omitted due to filtering time constraints. The mean difference in Cq-value and associated 95% CI of all qPCR replicates was calculated. All statistical analyses were performed using SPSS IBM Corp. (2013).

Results

SPECIES DETECTION

Altogether 713 qPCR samples, including controls, were analysed. No samples were discarded. Perch and pike were both detected in most of the qPCR runs from the study lake (314 of 365, Fig. 2). For both species, SX_{TUBE} showed the highest overall detection rate (95% perch and 96% pike) and EP the lowest (89% perch and 56% pike; overall difference $SX_{TUBE} \neq$ EP: Pearson χ^2 (1, n = 62) = 6·9, Fisher's exact P = 0·02).

CAPTURE METHOD

A CHAID tree multivariate predictive model was successfully generated from perch Cq-values. Capture method was the best overall predictor of Cq-values, better than storage media or storage time. In general, the lowest Cq-values were generated from $SX_{CAPSULE}$ samples in comparison with other capture methods (Fig. 3a). We validated the fundamental first-level outcome from this multivariate model for perch with new data in the build of a second CHAID tree, modelling pike Cq-values (Fig. 3b). In this second variant, capture was also the best predictor of Cq-values and $SX_{CAPSULE}$ tied with the CN and GF filters in the lowest value category.

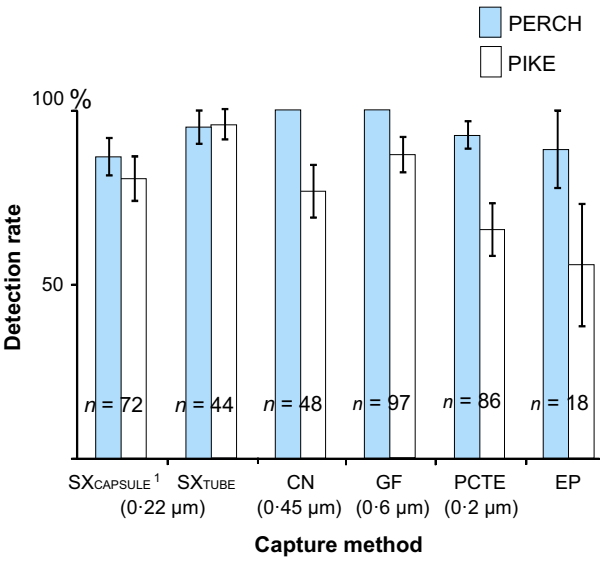

Fig. 2. Detection rate using quantitative PCR (qPCR; study lake). Blue bars and clear bars show positive detections of perch and pike, respectively. Pore size of filters within parentheses. $SX_{CAPSULE}$, Sterivex, extraction within filter capsule; SX_{TUBE}, Sterivex, extraction in tube outside capsule from removed preservation buffer; CN, cellulose nitrate; PCTE, polycarbonate track-etched; GF, glass fibre; EP, ethanol precipitation. Error bars represent standard errors; n indicates number of trials pooling all replicates for each method and both species combined. [1]Deviating from protocol, 12 $SX_{CAPSULE}$ replicates were over-vortexed and tested mainly negative. If these 12 over-vortexed samples are omitted, the detection rate estimate for $SX_{CAPSULE}$ increases to 100% for perch and to 91% for pike.

The fundamental first-level outcome of both the CHAID tree multivariate predictive models was supported in a one-by-one comparison of capture methods including both species and all treatments. Overall, $SX_{CAPSULE}$ was more efficient than the other capture methods apart from CN. $SX_{CAPSULE}$ yielded significantly higher [$eDNA_{tot}$] and lower Cq-values (Table 2). SX samples contained up to 118 ng total eDNA μL^{-1} and most $SX_{CAPSULE}$ amplified before 36 cycles (Fig. 4). [$eDNA_{tot}$] from the fish-free control pond showed a similar pattern, being higher for CN and $SX_{CAPSULE}$ compared with GF and PCTE (Mann–Whitney U = 12, $n_1 = n_2$ = 10, Fisher's exact P = 0·003), but with no Cq-values from qPCR as target species were not present. Overall, capture method and [$eDNA_{tot}$] were fundamental predictors of Cq-values (Fig. 4).

STORAGE PRESERVATIVE

SX-specific storage results are singled out and illustrated in Fig. 5. SX_{TUBE} samples treated with RNAlater, a significant predictor of poorer Cq-values in the CHAID trees, were least successful. For $SX_{CAPSULE}$, preservation in ethanol or Longmire buffer improved Cq-values for perch in comparison with frozen, 5 h and preservation in RNAlater (Figs 3a and 6). Also for both species pooled, these two buffers (ethanol or

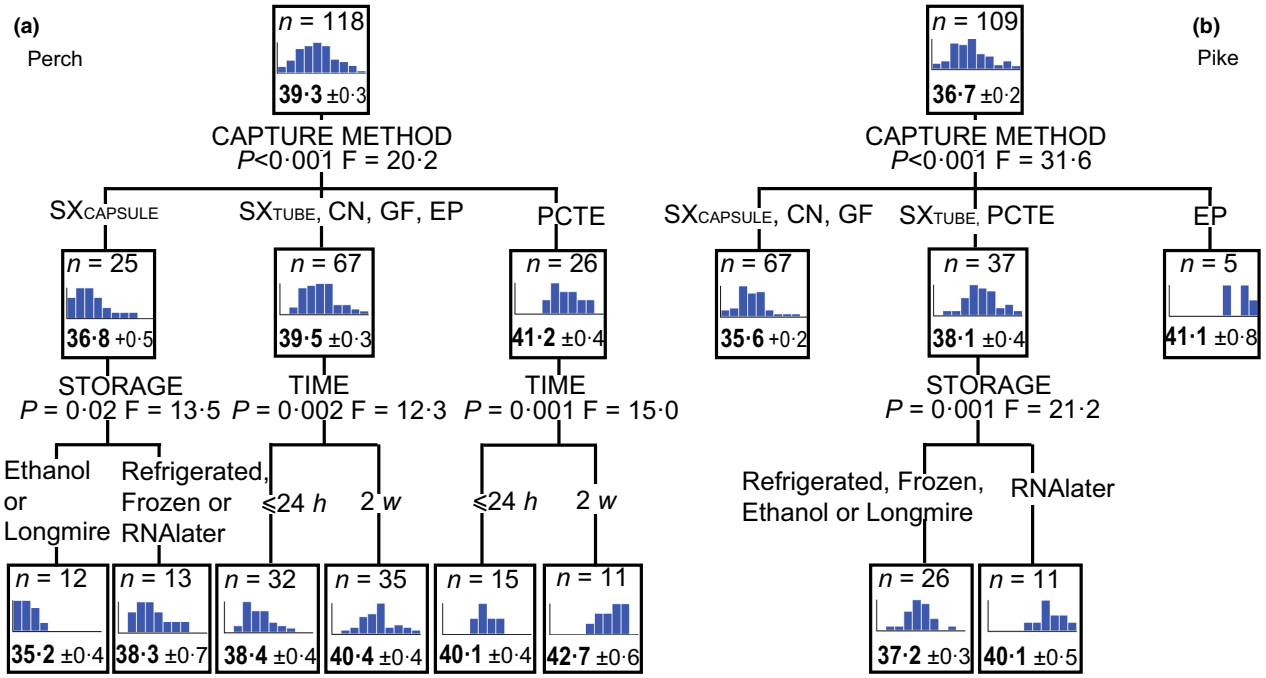

Fig. 3. Chi-square Automatic Interaction Detector decision trees relating three categorical variables (capture method, storage treatment and storage time) as model predictors for Cq-values as response target (study lake). (a) Perch. Best predictor was capture method, followed by storage time, and finally, storage treatment. (b) Pike. Best predictor was capture method followed by storage treatment. $SX_{CAPSULE}$, Sterivex, extracted within capsule; SX_{TUBE}, Sterivex, extraction in tube outside capsule; CN, cellulose nitrate; GF, glass fibre; PCTE, polycarbonate track-etched fibre; EP, ethanol precipitation; h, hours; w, weeks. Blue bar charts indicate relative size distribution of Cq-values within each category before split. Number under bar charts indicate mean Cq-value for the given category ± SE.

Table 2. $SX_{CAPSULE}$ in comparison with other eDNA capture methods

$SX_{CAPSULE}$ comparison of Cq-values ($SX_{CAPSULE}$ comparison of [$eDNA_{tot}$])					
Capture	Pairs of *n*	*P*	Significance*	*Z*	Rank
SX_{TUBE}	33 (18)	**1×10^{-5} (5×10^{-4})**	*** (**)	−4·4 (−3·5)	$SX_{CAPSULE}$ < SX_{TUBE} (>SX_{TUBE})
GF	50 (27)	**7×10^{-3} (2×10^{-5})**	* (***)	−2·7 (−4·3)	$SX_{CAPSULE}$ < GF (>GF)
PCTE	44 (27)	**1×10^{-5} (6×10^{-6})**	*** (***)	−4·4 (−4·5)	$SX_{CAPSULE}$ < PCTE (>PCTE)
EP	13 (9)	**1×10^{-3} (8×10^{-3})**	** (*)	−3·2 (−2·7)	$SX_{CAPSULE}$ < EP (>EP)
CN†	29 (15)	0·32 (0·55)	N.S. (N.S.)	−1·0 (−0·6)	

Wilcoxon matched-pair signed-rank test of both Cq-values from qPCR and [$eDNA_{tot}$] (denoted in parentheses). Significant *P*-values are in bold and non-significant *P*-values are denoted as N.S.

$SX_{CAPSULE}$, Sterivex, extracted within capsule; SX_{TUBE}, Sterivex, extraction in tube outside capsule; GF, glass fibre; PCTE, polycarbonate track-etched filter; CN, cellulose nitrate; EP, ethanol precipitation; [$eDNA_{tot}$], total eDNA concentration.

*Bonferroni corrected (5 tests): $\alpha = 0{\cdot}05$ lowered to 0·01, $\alpha = 0{\cdot}01$ lowered to 0·002 and $\alpha = 0{\cdot}001$ lowered to 0·0002.

†Due to time constraints, CN (24 h) were cancelled reducing sample size and statistical power for CN in comparison.

Longmire) in $SX_{CAPSULE}$ resulted in lower Cq-values compared with frozen or 5 h (Mann–Whitney Test *U*: 35, $n_1 = 23$, $n_2 = 15$, $Z = -4{\cdot}1$; $P = 4 \times 10^{-5}$).

STORAGE TIME

Storage time in the second-level outcome from the first CHAID tree was classified as a positively correlated predictor of Cq-values for all capture methods apart from SX (Fig. 3a). This was supported in a one-by-one comparison of capture methods including both species and 24 h to 2 weeks treatments (Table 3). Cq-values did not increase significantly with time using SX, but did with GF and PCTE.

The mean difference between Cq-values of paired qPCR replicates run within the same day was +0·3 ± 0·2 SE. This difference increased to +1·3 ± 0·2 SE when replicates run on different days were included, indicating that freezing and thawing of eDNA once or twice between measurements decreased DNA quality [Welch's test $t(1, 68) = 7{\cdot}1$, $n_1 = 20$, $n_2 = 80$, $P = 9 \times 10^{-10}$]. To avoid introducing this error, only DNA templates thawed for the first time were included when calculating average Cq-values for the samples.

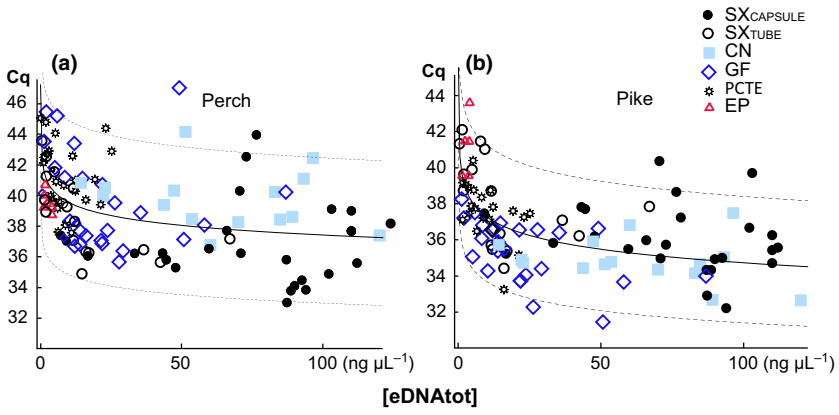

Fig. 4. Environmental DNA (eDNA) capture methods: relationship between total eDNA concentration ([$eDNA_{tot}$]) and quantification cycles in qPCR (Cq-value) in study lake. Line represents best-fit power function where Cq decreased as a function of [$eDNA_{tot}$]. (a) Perch. $Cq = 41{\cdot}8 \times [eDNA_{tot}]^{-0{\cdot}024}$; $P < 0{\cdot}001$, $R^2 = 0{\cdot}23$. (b) Pike: $Cq = 40{\cdot}0 \times [eDNA_{tot}]^{-0{\cdot}031}$; $P < 0{\cdot}001$, $R^2 = 0{\cdot}42$. Dotted lines represent lower or upper limits of 95% CI for slope of regression. $SX_{CAPSULE}$, Sterivex, extracted within capsule; SX_{TUBE}, Sterivex, extracted from buffer in tube outside capsule; CN, cellulose nitrate; GF, glass fibre; PCTE, polycarbonate track-etched fibre; EP, ethanol precipitation.

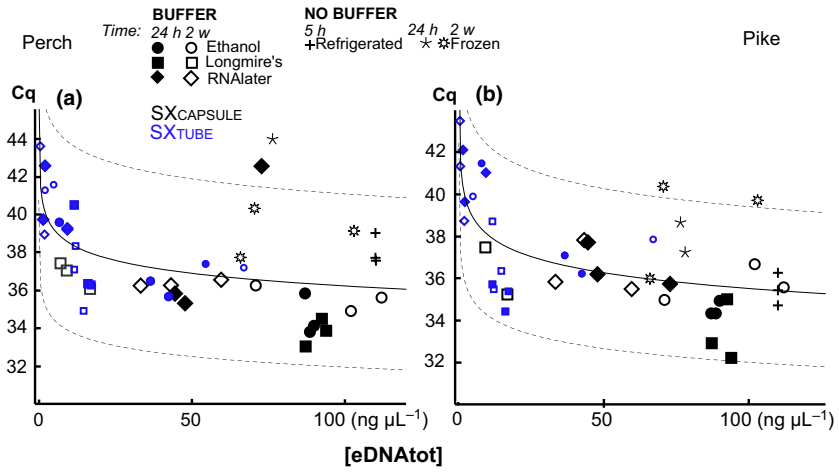

Fig. 5. Environmental DNA (eDNA) storage treatment using SX: relationship between total eDNA concentration ([$eDNA_{tot}$]) and quantification cycles in qPCR (Cq-value) in study lake. Line represents best-fit power function of the negative correlation between Cq and [$eDNA_{tot}$]. (a) Perch: $Cq = 40{\cdot}9 \times [eDNA_{tot}]^{-0{\cdot}026}$; $P < 0{\cdot}001$, $R^2 = 0{\cdot}28$. (b) Pike: $Cq = 40{\cdot}8 \times [eDNA_{tot}]^{-0{\cdot}030}$; $P < 0{\cdot}001$, $R^2 = 0{\cdot}45$. Dotted lines represent lower or upper limits of 95% CI for slope of regression. Sterivex, extracted within capsule ($SX_{CAPSULE}$) and from buffer in tube outside capsule (SX_{TUBE}) shown in black and blue symbols, respectively. h, hours; w, weeks.

CONTAMINATION

One false-positive signal for perch was detected at 42 cycles in an EP 'no-water' negative control. Remaining negative controls for capture/storage treatments ($n = 80$) and negative pond water ($n = 85$), NTCs ($n = 64$) and 37/40 tissue negative controls for species specificity did not amplify. The contaminated tissue control was replaced and showed no amplification. One extraction blank came up positive in one of the seven runs, but at a very high Cq of 46·2.

Discussion

To our knowledge, this is the first study comparing enclosed filters (SX) with commonly used eDNA capture and storage techniques. Similarly to other capture methods, SX can be used to target a wide range of macro-organisms successfully (using PCR, qPCR or NGS; Table S1), ensuring the generality of SX for surveys of aquatic biodiversity.

Specifically, SX with added preservation buffer (ethanol or Longmire's) is the optimal approach of the tested treatments in regard to [$eDNA_{tot}$] yield and detection sensitivity for target species. Other eDNA studies of macrobiota using SX (Keskin 2014; Bergman *et al.* 2016) did not apply preservation buffers. Although our study set-up was different, the lake sample results are consistent with the mesocosm experiment of Renshaw *et al.* (2015), showing that open CN filter and polyethersulfone filters (same material as SX in this study) were more effective than PCTE and GF. Additionally, we demonstrate that SX eDNA retains integrity over time, whereas eDNA from the open filters degrades significantly. These results suggest that SX eDNA is more effectively preserved, possibly due to the fact that it is considerably less handled by the user. The capsule may reduce risks of exposure to physical and biogenic stress as well as contamination, because capture, storage and extraction take place within the filter capsule. This, together with extended field usage possibilities, and higher eDNA yields, constitutes reasons to recommend enclosed filters before other capture methods.

CAPTURE METHOD

Based on our results, we reject H_0 hypothesis 1 stating that SX and commonly used techniques in our study are equally

effective, because $SX_{CAPSULE}$ yields the lowest Cq-values for perch (Fig. 3a). However, this is only partially validated in the case of pike (Fig. 3b), where $SX_{CAPSULE}$, GF and CN group together for the lowest Cq-values. Overall, $SX_{CAPSULE}$ yields higher [$eDNA_{tot}$] and generates better qPCR results than other capture methods, with the exception of CN. Our CN/SX comparisons are not as extensive as the SX/GF and SX/PCTE comparisons (Table 2). We show that higher levels of [$eDNA_{tot}$] are related to lower Cq-values of target species DNA (R^2 = 0·23–0·45, Figs 4 and 5) and therefore suggest measurements of [$eDNA_{tot}$] for approximate indications of eDNA capture efficiency.

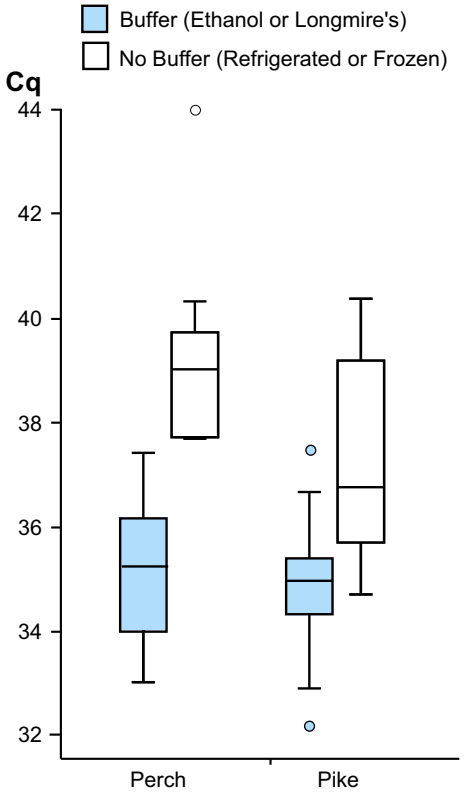

Fig. 6. Boxplots of Cq-values showing $SX_{CAPSULE}$ (extraction within Sterivex capsule) filter storage with and without preservation buffer (ethanol or Longmire's).

The comparison in this study of SX_{TUBE} to $SX_{CAPSULE}$ demonstrates that utilizing both these sources of eDNA should be useful. Pooling of these in the final elution step would be advisable for gaining even higher final yields of eDNA. SX_{TUBE} exhibits the highest overall detection rate for both species (95–96%) in our study, significantly higher than EP results. Higher amounts of false negatives from EP field samples may be due to DNA retention in the falcon tubes (Gaillard & Strauss 1998) and/or to the low water volume processed (0·015 L; Deiner *et al.* 2015; Eichmiller, Miller & Sorensen 2016; Minamoto *et al.* 2016).

STORAGE PRESERVATIVE

We reject H_0 hypothesis 2a stating that preservation buffers for storage of SX do not affect qPCR amplification in comparison with extraction within 5 h or freezing at −20 °C. Two-thirds of published aqueous eDNA surveys reporting storage details apply freezing of filters as a preservation method (Table S1 and S2), while less than one-third of surveys use buffer storage. Our results indicate that addition of ethanol or Longmire's immediately after SX filtration provides the lowest Cq-values, and is significantly better than freeze storage or extraction within 5 h. Based on our results as well as the results of three previous studies (Renshaw *et al.* 2015; Wegleitner *et al.* 2015; Minamoto *et al.* 2016), we recommend addition of preservation immediately after filtration.

STORAGE TIME

We reject H_0 hypothesis 2b that degradation of captured eDNA is the same in SX filters and the other capture techniques tested in this study. Cq-values increase significantly with storage time for GF and PCTE samples, indicating degradation of eDNA. In contrast, Cq-values for SX samples ($SX_{CAPSULE}$ or SX_{TUBE}) do not differ significantly after 2 weeks of storage at RT.

We note that repeated use of the same extracted eDNA sample (eluted in TE-buffer) for qPCR on different days, entailing repeated freezing and thawing, resulted in higher Cq-values. Freeze–thaw-induced degradation and/or inhibition of DNA is previously acknowledged (e.g. Ross, Haites

Table 3. Effect of storage time for eDNA results with different capture methods

Paired test of Cq-values					
Storage	Pairs of *n*	*P*	Significance*	*Z*	Rank
$SX_{CAPSULE}$	20	0·15	N.S.	−1·5	
SX_{TUBE}	16	0·18	N.S.	−1·3	
PCTE	16	**0·002**	**	−3·1	PCTE 24 h < PCTE 2 weeks
Glass fibre (GF)	24	**0·002**	**	−3·1	GF 24 h < GF 2 weeks

Wilcoxon matched-pair signed-rank test of Cq-values from qPCR. Storage 24 h paired with storage 2 weeks. Significant *P*-values are in bold and non-significant *P*-values are denoted as N.S.
Due to time constraints, cellulose nitrate treatments (24 h) were cancelled.
$SX_{CAPSULE}$, Sterivex, extracted within capsule; SX_{TUBE}, Sterivex, extraction in tube outside capsule; PCTE, polycarbonate track-etched filter.
*Bonferroni corrected (4 tests): α = 0·05 lowered to 0·0125, α = 0·01 lowered to 0·0025.

& Kelly 1990; Takahara, Minamoto & Doi 2015). We therefore recommend that extracted eDNA samples are divided into many aliquots immediately after extraction, in order to avoid compromising eDNA quality by repeated freezing and thawing.

CONTAMINATION

We cannot yet reject H_0 hypothesis 3 stating that SX leads to as many false positives as typically used methods. We only produced one false positive (EP) which is insufficient for any statistical inference. The SX approach using sealed pre-sterilized equipment until sampling, and capping filter immediately after filtration, should reduce contamination risk. The contamination variance between these capture methods remains to be tested using more observations and possibly synthetic controls (Wilson, Wozney & Smith 2016).

LIMITATIONS

The hand-held syringe used with SX filter units is convenient but turns into a labour-intensive bottleneck when processing many samples. This can be alleviated by switching to battery-powered pumps (Sterivex™ 2013). In 'algal soup' or turbid waters, 0·2 μm pore size may pose a problem as the filters clog easily and less water can be processed (Turner *et al.* 2014a). This can be overcome by pre-filtering (Robson *et al.* 2016) and/or increasing the number of filter replicates. Future research is needed to identify optimal procedures for highly productive and/or turbid waters.

Conclusion

In conclusion, we recommend SX filters as an efficient capture method for aqueous eDNA sampling of macro-organisms. Preservation of SX in ethanol or Longmire's buffer immediately after filtration is recommended. Preserved SX capsules may be stored at RT for at least 2 weeks without significant degradation. Water samples can be quickly filtered and preserved on site requiring less equipment, easing transport. Therefore, SX capsules are logistically compatible with remote and harsh field conditions.

Authors' contributions

M.H. and J.S. conceived and designed initial experiment. All authors (except D.H.) contributed to final design and participated in 'sample collection/filtration day'. J.S. analysed data and drafted the manuscript. M.H. developed protocol for eDNA capture/extraction. J.S., M.H. and A.E. wrote the manuscript. A.E. and S.S.T.M. coordinated field experiment and contributed to extraction protocol. A.E., M.H., S.W.K., S.S.T.M., E.E.S. and M.S. extracted DNA. S.W.K. optimized qPCR protocol. S.W.K., M.H. and M.S. performed qPCR assays. All authors revised the manuscript. No conflict of interest exists.

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Data accessibility

Data are deposited in the Dryad Data Repository http://dx.doi.org/10.5061/dryad.p2q4r (Spens *et al.* 2016).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Flow chart illustrating the different capture and storage treatments.

Appendix S1. eDNA extraction protocol.

Appendix S2. Water quality in Gentofte lake.

Table S1. Empirical field-studies targeting macrobial eDNA in aquatic ecosystems with water sampling, January 2005 to March 2015.

Table S2. Empirical field-studies targeting macrobial eDNA in aquatic ecosystems with water sampling, published after the current study was initiated in March 2015.

Table S3. Primers and probes used in this study.