An evidence review for great crested newt eDNA monitoring protocols

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Foreword

DNA based applications have the potential to significantly change how we monitor and assess biodiversity. *Triturus cristatus* or great crested newt (GCN), is an example of a fairly cryptic pond species, with a relatively low detection rate using traditional methods of sampling, which are resource intensive. A project was carried out in 2013/14 to establish the performance of environmental DNA techniques for determination of the presence of GCN in a wide variety of pond habitats across the United Kingdom. As part of this project a technical advice note (Biggs et al. 2014) was developed which contained the eDNA field and laboratory methods to be used for the detection of GCN using eDNA for use from the 2014 GCN season onwards.

Natural England wanted to review this protocol and consider if more recent changes in technology would be appropriate. This project had 6 desk-based tasks, with no new field work carried out:

1. Compare the effectiveness of the ethanol precipitation and filtration eDNA capture methods for GCN
2. Evaluate the field protocol for collecting and processing the water samples; including the use of single-use plastics, and how this can be minimised and also use by dates for kits subject to appropriate storage regime. Recommend any changes to the current field protocols supported by relevant evidence
3. Evaluate the laboratory procedures specified in the current protocol (WC1067) in light of developments since 2014; and recommend any changes to the current protocols supported by relevant evidence (this could include different potential changes to the same part of the protocol)
4. Via consultation create a list of any projects underway looking at GCN protocols and/or methodologies
5. Consider areas of the current protocol where flexibility could be allowed rather than being fixed, whilst still maintaining appropriate assurance in methods and results
6. Conduct a cost-benefit analysis of any proposed changes to the methodologies (field and laboratory), compared to the existing protocol

The findings of this report will now be reviewed, both internally and in consultation with interested parties, and the next steps decided. No immediate changes are being made to the existing protocol, which continues to be the accepted methodology for this work.

Natural England commission 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

DNA based applications have the potential to significantly change how we monitor and assess biodiversity. *Triturus cristatus* or great crested newt (GCN), is an example of a fairly cryptic pond species, with a relatively low detection rate using traditional methods of sampling, which are resource intensive. A project was carried out in 2013/14 to establish the performance of environmental DNA techniques for determination of the presence of GCN in a wide variety of pond habitats across the United Kingdom. As part of this project a technical advice note (Biggs et al. 2014) was developed which contained the eDNA field and laboratory methods to be used for the detection of GCN using eDNA for use from the 2014 GCN season onwards. Now with the experience of 9 year's-worth of GCN surveillance by eDNA Natural England wishes to review the available evidence to compare the effectiveness of ethanol precipitation and filtration eDNA capture methods for GCN and to evaluate the field and laboratory protocols within the technical advice note for recommendations and any changes in the current protocols that could be made in light of more recent technological developments and whether any of the proposed changes would result in any cost-benefit.

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1. Introduction

Environmental DNA (eDNA) can be described as the DNA trace left behind in an environment (land, sea or air) where an organism has once been (Taberlet et al. 2012, Rees et al. 2014, Pawlowski et al. 2020). This trace DNA is likely to be deposited through secretion (saliva), excretion/defecation (skin cells, urine and faeces), and the release of gametes into the environment. The detection of species-specific DNA sequences within environmental samples by both PCR and by DNA sequencing technologies (metabarcoding) is revolutionising how species monitoring within the environment can be carried out. From its early description only 15 years ago (Ficetola et al. 2008) eDNA methods have now been widely adopted and are a valuable tool for species monitoring effort. Natural England was an early adopter of the technique and demonstrated that inclusion of eDNA methods into great crested newt (GCN) surveys could have potential to improve survey effort by speeding up burdensome survey time associated with population survey and then the mitigation steps required if GCN populations are detected. The adoption of eDNA methods for GCN detection came from supporting data funded by Natural England and described by Biggs et al. (2015) who demonstrated a strong agreement between eDNA detection and conventional survey results, concluding that 'eDNA is a highly effective survey method and could be used as the basis for a national great crested newt monitoring programme'. Since 2014, tens of thousands of GCN tests have been carried out in the UK following the methodology published as a technical advice note WC1067 that was peer reviewed by a panel of experts. Several commercial labs have entered the market that offer seasonal GCN testing for ecologists in support of this survey effort. This methodology has remained unchanged, allowing labs to run a validated method to known sensitivities which has high alignment with detection of GCN in the environment (using traditional observation and trapping methods). Since 2017 an externally managed proficiency testing scheme has been run by FAPAS® so that suppliers can monitor their assay consistency for GCN eDNA service provision.

Since the introduction of GCN eDNA testing and the agreement of the Natural England methodology several methodological improvements have come to the fore, some of which could now be regarded as standard within the field of eDNA based research. As the current method has been in place for over 9 GCN survey seasons Natural England have commissioned this report to summarise the evidence that is currently available on any methodological advances and its application with specific reference to GCN eDNA testing.

One of the areas that has seen the biggest change is in how the samples are collected, and the extensive description in the scientific literature on the use of filter based eDNA capture technology for the sampling of eDNA from waterbodies. The Natural England methodology uses an ethanol precipitation of eDNA, this was one of the first methods described for eDNA recovery (Ficetola et al. 2008, Biggs et al. 2014) and was incorporated into the early sampling plans that were used for the Natural England trials that developed the current eDNA protocols. Since the introduction of these methods, filtration has largely replaced ethanol precipitation as the method of choice for capturing eDNA from water samples. This methodology offers several advantages over ethanol precipitation, these,

and the possible caveats for its use (in application for GCN monitoring) are presented within this report.

This report summarizes the data that is available with respect to GCN monitoring answering 6 specific tasks and subject areas that were specified by Natural England.

2. Methods

Literature Review

To fully understand how filtration methods would compare to ethanol precipitation in the context of GCN surveillance, we have looked to gather any evidence that has been presented over the last 8 years since the technical advice note was published. This study set out to perform a targeted literature review that could provide any evidence on these different eDNA sampling methods in relation to GCN monitoring and eDNA detection. The databases used were: Web of Science; and Google Scholar. Grey literature was sourced by applying search terms to the Google search engine.

The following terms were used: “GCN”; “Great Crested newt”; “eDNA”; “environmental DNA”; “DNA”; “filter”; and “filtration” in various combinations.

In anticipation that we would find low number of studies, we also looked to summarise the available data that has been presented for the two methods that may have been used for other species eDNA studies where we could find them.

Evaluation of the field protocol for collecting and processing water samples

We, and others, have generated data demonstrating that eDNA kit shelf life could be extended to the whole season rather than the currently used 3 months from addition of the DNA marker for the degradation control. This data was re-visited along with further studies carried out by Surescreen and NatureMetrics (information supplied via an Office 365 form sent to GCN eDNA service providers) that have looked at longevity over longer periods of time. It is likely that there can be substantial waste savings by increasing the shelf life of the ethanol based eDNA sampling kits to further lengths of time. Whilst ADAS have tried to minimise the use of single use plastics for the sampling of water- including the use of recycled packaging, there is scope for improvements, and this has been considered in the evaluation.

In order to understand, from their experience in field, how the field protocol works in practice and where there may be scope for improvements, we canvassed the expertise of ecologists with first-hand experience of GCN ecology and eDNA sample collection. RSK ADAS employed ecologists and several of our eDNA client’s ecologists were invited to provide answers to a questionnaire aimed at understanding how the in-field protocol works in practice and where there may be scope for advancements. We canvassed the opinions of GCN licenced ecologists via an anonymised Office 365 forms survey consisting of 16 questions. A summary of these questions is detailed in Appendix 3.

Evaluation of the laboratory procedures specified in the current protocol (WC1067) in light of developments since 2014.

For this evaluation we relied on our own expert knowledge of the procedures involved within the technical advice note and literature relating to eDNA analysis published since 2014. RSK ADAS Ltd have been a supplier of GCN eDNA analysis since 2014 and H. Rees was involved in the peer review of the technical advice note prior to its publication. Furthermore, RSK ADAS Ltd have scored 100% in every year of the proficiency testing run by FAPAS® on behalf of Natural England making us experts in the field. Further information was sourced from web searches using the Google search engine using terms such as: DNA extraction kit, PCR mastermix, PCR inhibitor removal kit amongst others. Recommendations for changes to the procedures were made after a full evaluation of the evidence available.

GCN protocols and/or methodology Projects

To answer this question an Office 365 Forms survey was produced and sent to the following GCN eDNA service providers: Applied Genomics; Surescreen; FERA; NatureMetrics; Cellmark; and previous service provider Spygen. The questions asked are detailed in Appendix 6.

Areas of flexibility

The evaluation of the current protocols detailed in the technical advice note were reviewed with respect to the potential advances made in eDNA detection from water sources since adoption of the GCN eDNA detection. Potential areas of the methodology where flexibility could be introduced to achieve a more cost-effective, environmentally friendly and streamLined process, are listed.

Cost-benefit analysis

The cost benefits (or otherwise) of alternative approaches were tabulated in excel format with an identification of the where cost savings may be made. The cost benefit of some changes could be used to offset the higher cost of using items that are ultimately recyclable or not single use.

For the sampling kits we considered what cost savings could be incurred by using alternatives to the current methodology i.e. the cost associated with the supply (and return) of the current eDNA sampling kit and the likely saving that alternative kit components may bring. Prices, correct in September 2022, were obtained from various suppliers including: Fisher Scientific, Avantor, Merck and others. As staffing costs will

inevitably differ between service providers, we did not consider the costs of procurement or the costs associated with labour and the assembly of the sampling kits.

For the laboratory procedures we do not deal with labour or overheads and only consider cost-benefits to do with the purchase of laboratory consumables.

For the surveyor/service user we considered the potential cost savings incurred by using alternative consumables both within the sampling kits and in the laboratory and whether these can be passed on.

Task 1. Compare the effectiveness of the ethanol precipitation and filtration eDNA capture methods for GCN.

Ethanol precipitation as the method of recovery of eDNA from water samples was adopted in 2014 within the Natural England technical advice note (WC1067) after extensive validation of a PCR assay that was initially described by Thomsen et al. (2012) for GCN detection, in conjunction with DNA collection methods based on earlier works by Ficetola et al. (2008). The ethanol precipitation method acts both as a preservative to keep DNA intact before analysis (guidelines are for storage of collected samples for up to 1 month before analysis) and (in the presence of sodium acetate) acts to neutralise the negative charges on the phosphate residues of DNA and raise the hydrophobicity of the DNA which results in its increased propensity of DNA to fall out of solution. This ‘precipitated’ DNA can then be recovered by a simple centrifugation which will recover both ‘free’ or ‘acellular’ DNA and also results in the recovery of cellular/organelle associated DNA, the likely state most eDNA will start as (Harrison et al. 2019). Ethanol preservation/precipitation has been shown to be a reliable method for the recovery of eDNA but offers poor scope for the large sample volumes necessary where there may be a need to increase the rate of detection of low abundance target DNA. For example, those associated with extremely dilute eDNA concentrations particularly populations of animals in flowing water or in larger water bodies such as lakes.

The Natural England technical advice note could now be seen as being an unusual method for DNA sampling that though effective is perhaps out of step with current eDNA methods which have largely moved over to filtration-based sampling. All this despite the method being the most highly validated eDNA assay that is currently being used in a regulatory framework (Thalinger et al. 2021). A number of different filter technologies have now been described (Turner et al. 2014, Wilcox et al. 2015, Hosler 2017, Deiner et al. 2018, Sepulveeda et al. 2019 etc.), with variations in membrane filter chemistry, pore size, filter size and whether these are used in an open or closed filter system. Pore sizes of around 0.45-0.8µm are regarded as ideal for most eDNA capture protocols, 0.2µm often being adopted when microbial community analysis is also of interest (Lee et al. 2010). Filtration methods usually rely on the hand filtration of water via the use of large syringes

to push water through filters, removing the reliance and complications associated with pump-based systems, especially when used in field. Once samples have been collected there is still a requirement to preserve any DNA containing material on the filters and a variety of preservation solutions and buffers have now been described, ethanol or Longmires solution being the most popular (Longmire et al. 1997, Wegleitner et al. 2015, Bruce et al. 2021). Filter systems have the advantage that much larger sampling volumes are achievable than can practically be obtained with ethanol precipitation, this greater sample volume can result in a concomitant increase in the detection rate of target DNA but much of this will be dependent on the quality of the water and currently there is a lack of studies using pond water (discussed in Harper et al. 2019) with filtration generally being applied to rivers, lakes and experimental systems.

To fully understand how filtration methods would compare to ethanol precipitation in the context of GCN surveillance, we have looked to gather any evidence that has been presented over the last 8 years since the technical advice note was published. To gather this evidence a literature search was a carried out to summarise any evidence that has been described in relation to GCN monitoring and eDNA detection. The following terms were used to search Web of Science and Google Scholar: “GCN”; “Great Crested newt”; “eDNA”; “environmental DNA”; “DNA”; “filter”; and “filtration” in various combinations. As of July 2022, a large number of papers were identified but further screening of the titles and abstracts of these papers suggested that only one paper was directly relevant to the search. This study was carried out by Buxton et al. (2018a) which compared filtration with ethanol precipitation for the recovery of GCN eDNA from both ponds and experimental tank systems with known populations of GCN. Whilst filtration was seen to outperform precipitation in tank models containing relatively pure water these results could not be replicated when pond water was tested. There was no discussion of potential inhibition of the PCR assay which could result from the increased volumes of water sampled. It should be noted that between 5 and 10x the volume of water was filtered than was captured by ethanol precipitation. The paper highlights the difficulty of passing pond waters through filters and suggests that the higher turbidity of pond water (compared to other fresh water sources), may not be appropriate for GCN sampling from ponds. *‘*Pond water can differ from water found in rivers, lakes, or the marine environment. Pond water is more stagnant, allowing the build-up of algae and suspended solids to a greater extent than lotic water or large lakes where stratification and wind action allow for water movement*.’* The authors suggest that in their hands the results that they obtained in their pond field experiments were not fully supportive of other pieces of work that suggest filtration outperforms precipitation (Deiner et al. 2015, Spens et al. 2016). It should be noted that this comparison of filter and ethanol-based techniques did not look to evaluate the plethora of different filters that are on the market (size, chemistry and pore size). Other variables such as pre-filtration either by a tandem glass fibre larger pore filter or by a filter employing a pre-filter may be less prone to clogging and more efficient at capturing eDNA than that described (Bruce et al. 2021).

The apparent lack of data available on the comparative use of filter versus ethanol precipitation for pond water analysis is highlighted by Harper et al. (2019), who again

report that filtration methodologies are generally associated with higher filtration volumes and higher sample throughputs with an increasing potential to recover greater amounts of DNA (Spens et al., 2016; Hinlo et al. 2017; Klymus et al. 2017). A point highlighted in this review is that these studies generally exclude ponds and make comparisons for water from rivers, lakes, and experimental aquaria, which are generally of far higher water quality than that sampled from ponds. Ponds often contain levels of suspended solids and algae, and water quality deteriorates over summer months with ponds drying out due to evaporation and/or suffering from increased levels of algae associated with high levels of sunshine. They point out that under these circumstances water filters can clog with only very small water volumes (Klymus et al., 2017; Raemy & Ursenbacher, 2018) and under these circumstances protocols that include ethanol precipitation or extensive pre-filtering of water are likely to be more useful. There are no further published methods that compare filtration to ethanol precipitation in the context of GCN eDNA analysis.

In addition to published studies, unpublished research conducted by RSK-ADAS on a limited number of ponds known to contain GCN populations has been carried out to compare three commonly used filter types with ethanol precipitation using a total volume of 90mLs water (the volume of water currently sampled and analysed in accordance with the technical advice note) to allow for direct comparison of the different capture methods (highlighted in answer to Task 4). In each case the mean Ct values of the filter sampled waters were between 1.2 and 4 values lower (mean of 2.6 Ct) than the Ct values from the same water that had been ethanol precipitated (Appendix 1). This would suggest that on average filtration recovered at least 5x the yield of target GCN eDNA than ethanol precipitation, when comparing the same water samples and same volumes of water. The caveat to this study was that the ponds tested were known to contain high populations of GCN and were of low turbidity. This preliminary data does however suggest that to demonstrate equivalence to the current methodology you do not need to filter large volumes of water.

Additional data (in preparation for publication) provided by NatureMetrics with Atkins and HS2 Ltd (Appendix 2) compared the effectiveness of filtration with ethanol precipitation for GCN eDNA capture. The water sampling protocol from the technical advice note was used for both eDNA capture methods with minor modifications for filtration, where 20 x 125 mL subsamples were collected at equidistant intervals around the pond perimeter and pooled into a single sampling bag for homogenisation, following which as much water as possible was filtered. The results showed that GCN eDNA was detected by both methods from April to July (the eDNA survey season lasts mid-April to end of June) although not all previously positive ponds were positive for GCN in every month they were sampled. Filtration produced more GCN positive ponds than ethanol precipitation from April to June, but ethanol precipitation produced slightly more GCN positive ponds in July. This finding of GCN eDNA in July matches those of previous studies (Rees et al. 2017 and Buxton et al. 2018b, discussed in Task 2). The average eDNA qPCR score across ponds was higher using filtration than ethanol precipitation each month. This is not unexpected given that as much water as possible was filtered making it likely that more GCN eDNA was recovered than was possible with the ethanol precipitation kits.

The filtration method produced a positive (1 out of 12) score for a pond with a previously negative GCN status. This may be an example of where filtration of higher water volumes can be more sensitive than the ecologist performing traditional survey (discussed above).

A few direct comparisons of ethanol precipitation and filtration methods have been published but are outside the context of GCN monitoring. Spens et al. (2016) compared different filter systems with ethanol precipitation for the detection of pike and perch eDNA from lake water. Ethanol precipitation had the lowest rates of detection when compared to five other filtration systems- although for one species was actually very similar to the filtration methods. Using tank experiments Minamoto et al. (2016) compared different polycarbonate filters with ethanol precipitation. Using the common carp as the eDNA target, and water from aquaria, the authors demonstrated that ethanol precipitation yielded the greatest number of eDNA copies and suggested that ethanol minimized the loss of eDNA during sample collection. The use of polycarbonate filters in increasing pore size reduced the sample collection efficiency. The authors point out that in contrast to their experiments the concentration of target DNA in field samples is likely to be much lower and that the large volumes of water required for detection of common carp would make the use of ethanol precipitation unsuitable. In their field studies, ethanol precipitation showed the lowest levels of eDNA copy number, but this was down to the volumes of water that could be processed using ethanol compared to filtration. A study described by Deiner et al. (2015), compared different methods for eDNA capture/extraction and how these affect biodiversity assessment by both barcoding and metabarcoding methodologies. Analysis was carried out on equivalent volumes of water sampled by either filtration or precipitation. Total eDNA recovery was more influenced by the downstream processing method for DNA recovery than the method used for eDNA capture (ethanol precipitation or filtration), although this is less of an issue for GCN because downstream processes are standardised. However, the different protocol combinations for capture and extraction of eDNA significantly influenced DNA yield and number of sequences obtained from next generation sequencing. A study by Troth et al. (2020), compared ethanol precipitation and filtration for the detection of white clawed crayfish in mesocosms, ponds and rivers. Filters proved much better in a controlled mesocosm setting, and in a low population level pond setting, but interestingly in a river the ethanol precipitation performed slightly better. The authors highlight that there is not a one-size fits all eDNA sampling method and that local conditions may dictate best practice.

To fully answer this question, it is also important to understand the other advantages and disadvantages of each system which are important considerations if any methodological change is to be implemented (Table 1). There are certain advantages to the use of filtration such as the ability to extract from larger volumes of water (with an effective increase in sensitivity). Ethanol can be difficult to procure, store, ship and can cost more than filtration (largely down to couriering costs and the safe disposal of waste ethanol which is then recycled). Ethanol based kits need to be couriered following ADR regulations (a European agreement concerning the international carriage of dangerous goods by road) meaning that they must be shipped as dangerous goods in limited quantity incurring a greater cost. This can be especially costly for individual and low numbers of kits that need

to be couriered (see Task 6. Cost-benefit analysis). Filter sampling kits on the other hand are easier to prepare, ship and store in bulk (they are not flammable), can filter larger volumes of water, do not need centrifugation (costly equipment) and have gained widespread acceptance as the method of choice for eDNA sampling. The caveat to this is that large scale procurement of eDNA filter devices (especially the universally accepted Sterivex filters) is prone to long manufacturing and delivery times and these filters are prone to clogging with the types of water that typically come from UK ponds in early summer (those which are turbid), whereas ethanol collection is usually fine. Any move to allow the use of filtration within GCN monitoring would also require further training of surveyors (see Task 2).

Table 1. Advantages and disadvantages of ethanol precipitation and filtration methods.

Table 1. Advantages and disadvantages of ethanol precipitation and filtration methods.	Filters kits	Ethanol kits
Familiarisation	Ecology industry would require retraining to take samples	Ecology industry very familiar with the taking of samples
Validation	Not sufficiently validated in all pond water types	High level of validation over 10 years
Sample Volume	High volumes of water can be filtered 0.5-1L	Limited amounts of water 90mLs
	Greater volumes of water not necessarily an advantage- GCN eDNA needs to be 'tuned' to GCN detection by ecologist- if population survey is required.	
Sampling kit	Simpler sampling kit assembly	More complex sampling kit assembly
	Easier to ship (No ADR requirements)	ADR requirements can make shipping costly
	No standardised filter for use	Validated standard methodology
	Easier to store in bulk	Ethanol storage requires flammables cabinets
Cost	Similar sampling kit cost	
	Low shipping costs	Shipping costs can be high for low kit numbers
Water quality	Good for higher quality water, no problem using high Ca^{2+} water	Validated with a range of pond waters, not good with high Ca^{2+} water

Table 1. Advantages and disadvantages of ethanol precipitation and filtration methods.	Filters kits	Ethanol kits
Waste	No associated costs with waste collection	Waste ethanol requires specialist for disposal
	No large saving on plasticware waste filter and ethanol kits create similar amounts of plastic waste	
Procurement	Components relatively easy to procure, some filters have longer lead times	Ethanol can at times be difficult to procure in quantity
Throughput	Similar time required for DNA extractions from both capture method	
Proficiency test (Pt)	Unlikely to be compatible with current proficiency test (free DNA)	Compatible with current proficiency testing regime

Summary

Filtration technologies are now the method of choice for eDNA sample collection for studies involving collection from both lentic and lotic water sources. Filtration protocols, with options of incorporating a pre-filtration treatment in areas with high turbidity matrices, has allowed groups to filter greater volumes of water, improving eDNA yields and detection sensitivities when studying community biodiversity. We (RSK ADAS) have trialed filtration methods for GCN eDNA on ponds with good quality water and high GCN eDNA load. Our data suggested that all three filter types tested gave at least a 5-fold increase in amount of DNA than the same volume of water precipitated with ethanol. Although filtration would ultimately simplify the process of sampling kit assembly, shipping and handling, we do not have any evidence to compare like for like water samples that have been collected with both ethanol and filtration to demonstrate equivalence with all water types and qualities (turbidity).

To sanction the use of filters without this data could undermine previous monitoring effort. It is often suggested that filtration could allow greater volumes of water to be filtered and assayed increasing the likelihood of detection. This is true, but a caveat must be applied to the detection of GCN where data is to be used for early stage GCN mitigation (rather than District Level Licensing (DLL)) where the presence of an eDNA positive would need to be backed up by survey effort. If an increase in sensitivity results in the detection of trace amounts of sequence from either transient populations of GCN, or very low non-breeding populations, this sensitivity may well be out of line with the sensitivity of the ecologist who will be in field attempting a population survey. At present the ethanol precipitation of water

is well aligned with the sensitivity of the ecologist and the techniques used on the ground. On the evidence available comparing ethanol precipitation with filtration, filtration would be very appealing, offering a low-cost high throughput alternative to ethanol, which could be regarded as far more unwieldy to use. However, some points for consideration need to be highlighted:

- Evidence shows that filtration of eDNA can be carried out with multiple filter types and pore sizes, and each will capture a different fraction of the total eDNA in any water sample.
- There is therefore little standardisation in filtration techniques.
- Pond water is far more likely to clog filters at low sample volumes without extensive pre-filtration (this is not required with ethanol), an extensive range of pond water types and at different points in the season would have to be analysed using filtration to demonstrate equivalence with current ethanol precipitation methods.
- As pond water moves into late GCN season, water quality falls- there are higher amounts of algae and macroinvertebrates that are likely to cause issues with clogging filters than earlier in the season.
- eDNA analysis using the ethanol precipitation method has a high level of validation, due to its continued use over that last 9 GCN survey seasons.
- Although currently only 90 mL of water are sampled, the results generated for GCN eDNA is well tuned to the ecological survey in that when newts are present by trapping and survey, they can be detected by eDNA and vice versa. As is the case for early stage GCN license mitigation.
- Any perceived increases in sensitivity afforded by extraction from greater sample volumes could: 1) undermine previous survey efforts by detecting low/negligible GCN presence in ponds that were previously negative; and 2) increase the detections of eDNA from those low transient populations of GCN that are not picked up by traditional survey meaning that more site surveillance and wasted ecological survey effort could be put into chasing small non-breeding GCN populations where it would be extremely difficult to define a population. The sensitivity of any filtration method would therefore need to be tuned to that of ecological survey as done for precipitation method in the 2014 Defra report (Biggs et al. 2014).
- Whilst an argument for filtration is an increase in sensitivity of detection it is worth asking the question ‘is this required?’ given that the current methodology was shown to be equivalent to traditional survey (Biggs et al. 2014).
- The necessary rigour that Natural England has instilled in the testing labs by running the FAPAS® proficiency testing program would not currently be possible with a filtration-based method without further method optimisation. Free GCN target DNA (as opposed to cellular/organelle associated) as used in the FAPAS® proficiency testing scheme is unlikely to be captured by filtration-based methods (Trujillo-Gonzalez et al. 2021).
- This would also apply to the use of the synthetic degradation control DNA so its addition and composition could need to be standardised between laboratories.

Task 2: Evaluate the field protocol for collecting and processing the water samples; including the use of single-use plastics, and how this can be minimised and use by dates for kits subject to appropriate storage regime. Recommend any changes to the current field protocols supported by relevant evidence.

For this evaluation we have canvassed the expertise of ecologists with first-hand experience of GCN ecology and eDNA sample collection. RSK ADAS employed ecologists and several of our eDNA client's ecologists were invited to provide answers to a questionnaire aimed at understanding how the in-field protocol works in practice and where there may be scope for advancements.

The in-field sampling protocol was developed from extensive research undertaken within Natural England funded project WC1067 (Biggs et al. 2014). The Natural England technical advice note that was developed from this study forms the reference document, providing technical advice that must be adhered to by the service providers and the field staff, for both the collection and analysis of GCN eDNA samples. Adhering to this document has helped to ensure consistency within GCN eDNA detection surveys over the last 8 years. We canvassed the opinions of GCN licenced ecologists via an anonymised Office 365 forms survey consisting of 16 questions. A summary of these questions and the 78 responses that were obtained are detailed in Appendix 3 and included where relevant below.

Sustainability

The methodology for in-field eDNA sample collection uses ethanol precipitation kits, comprised of:

- A sterile 30mL ladle
- A sterile self-supporting Whirl-Pak plastic bag with 1 litre capacity
- A sterile 10mL pipette to resample the pond water
- Six sterile 50mL centrifuge tubes containing preservative (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents (Product Code: 10644795), sodium acetate and other markers)
- Two pairs of sterile gloves.

Each kit is assembled for the sampling of one pond i.e., is single use which is necessary to prevent any cross contamination of other water bodies. There is therefore no scope to reuse the components contained within the kit for additional water sampling. However, there are opportunities for more cost-effective and/or recyclable alternatives which could also reduce the amount of non-recyclable waste. The current methodology requires that sampling consumables should be 'DNA-free'/sterile, which not only requires extra processing and cost for this certification, but also means that each item is individually wrapped/sealed in paper or plastic. The qPCR for GCN detection is well validated and unlikely to amplify DNA from other species (Rees et al. 2014). With an assay that is species-specific, the need for 'DNA free'/sterile consumables is largely redundant provided that the kit contents are prepared in an environment that does not handle GCN DNA (also see comment on DNase free ethanol below). There could therefore be a reduction in the amount of packaging and non-recyclable packaging waste associated with the items that have been certified DNA free/sterile if this requirement was dropped. Withdrawing this requirement should remove the individual packaging waste associated with the ladle, pipette, and gloves.

With increasing awareness of sustainability and recycling it is important for all service providers to play their part to try and source components that are as environmentally friendly, plastic free or as readily recyclable as possible. RSK ADAS have mixed experiences of trying to source suitable suppliers that have stock of the required consumables at the necessary unit levels for the whole GCN season. Often service providers are subject to long delivery times and can incur additional cost sourcing suitable alternatives. Covid and the on-going effects of the UK exiting the European Union, continue to severely impact on supply and cost of plasticware required for eDNA sampling and analysis and often sourcing of the items has been more about purchasing what was available rather than sustainability.

In the survey ecologists were asked; 'Would clients be prepared to pay more for a GCN sampling kit, if all the consumables were guaranteed 100% recyclable?' Survey results (Appendix 3) show that 65% of samplers strongly agreed and 28% agreed that they would be willing to pay a price increase. Only 1% disagreed and 5% neither agreed nor disagreed, indicating that most clients would be prepared to pay more for readily recycled consumable items. We suggest that all laboratories offering a GCN detection service should be aiming to minimise the use of plastics. Where these alternatives are not possible, then a drive to find PET plastics should be made- these plastics are highly and readily recyclable. In our survey, when surveyors were asked how much of the sampling kit they currently recycled, responses ranged from none (21%) to all or as much as possible (30%), with several responders highlighting that they only recycled what they were sure could be (cardboard and some plastics). Some responders stated that components would be reused on other (non-eDNA projects) and within education. The biggest barrier to recycling some of these items appears to be knowledge of what can be recycled i.e. lack of sufficient labelling and the ease of which items that could be recycled can be dropped off at the appropriate recycling centre or collection service. It is suggested

that providers could make it easier for the end-user to understand which of the kit components could be recycled.

Each component of the sample collection kit is discussed in turn with respect to where more sustainable options could be made available.

Gloves

Providers are currently requested to add two packs of gloves per sampling kit. Gloves are required to be always worn during the sampling process, being replaced after sample collection from the pond and before pipetting to sterile sub sample tubes. Acute supply issues around sourcing gloves during the pandemic lead service providers to ask Natural England to accept the provision of one pair of gloves per sampling kit- these being worn throughout the water sampling process. We can confirm that since 2020 the supply of one pair of gloves per sampling kit has not caused any problem for ecologists and the requirement for the second pair of gloves in the current protocol should be removed. Sampling gloves do not need to be sterile and only need to be of low specification/standard rating, not medical or industrial grading as would usually be the case for the sterile gloves required by the technical advice note. The main purpose of wearing the gloves is to help prevent cross-contamination between sampling areas, and as PPE for the user from any direct skin exposure to water or spilled preservative solution. They are only worn briefly before being discarded, and there are several alternatives on the market (see Task 6, Table 2), where gloves are made from a plant-based raw-material suitable for composting. It is our opinion that there is little requirement for these gloves to be sterile (individually wrapped), therefore further reducing costs and packaging waste.

Pipettes

Pasteur pipettes are currently used to transfer 15mL of water sample from the collection bag to each of 6 sub sample tubes. Our questionnaire put forward the idea of utilising the ladle dipper for water transfer to the tubes, thus omitting the pipette from the sampling kit altogether. 59% of responders strongly agreed or agreed with this suggestion. However, 40% disagreed or strongly disagreed, illustrating that surveyors were divided. The transfer pipette does ensure consistency in the volumes of water transferred as pouring accurately from the sample dipper could be seen as challenging to carry out accurately. Again, transfer pipettes could be supplied as non-sterile and therefore not individually wrapped and can be purchased for measuring a range of volumes. Using smaller volume pipettes would also result in less plastic waste but would increase the workload of the ecologist, and the time taken to sample each area. The question was posed ’Would you be happy to use a smaller transfer pipette to save on plastics?’ 64% said that they strongly agreed, and 22% agreed that they would not mind the reduction in pipetting volume if it meant less plastic. These pipettes can be sourced from many of the major laboratory suppliers (Fisher, Appleton woods, VWR), and can be made from fully recyclable plastics for example low density polyethylene (LDPE). In general, laboratory plasticware is not marked

as being recyclable so even if items are made of fully recyclable plastics, we are uncertain how these would be identified in the downstream processing of domestic waste recycling.

Sample dippers

Sample dippers are currently supplied individually wrapped, with the outer bag having potential for use as the water collection bag in place of a separate sterile water sampling bag. 74% of ecologists strongly agreed and 19% agreed, that this was a viable alternative to using a separate bag. 4% either disagreed or strongly disagreed with this as a modification to protocol. Potential drawbacks from using the dipper bag for sampling, are that they are not as robust (made of thinner plastic), may not hold as large a volume as the current bags, and may not have a re-sealable top. We suggest that this potential change would need to be fully trialled by ecologists in the field to determine ease of use and the robustness of the packaging bag for this purpose. As with the other items in the sampling pack, there is no real reason why these items should be supplied certified sterile, and any relaxation to this specification would allow cheaper alternatives to be used.

50mL tubes

The ethanol collection tubes must meet certain specifications for suitability of use in the laboratory DNA extraction such as an ability to tolerate centrifugation speeds of 14,000 x g without fracturing or stress-leaking. Again, it is not necessary for these tubes to be certified DNA-free/sterile, a relaxation of this specification would allow service providers the option of having tubes supplied racked (in cardboard or polystyrene racks) or unracked within bulk bags. There are no suitable alternatives to plastic centrifuge tubes on the market, but there are options for sourcing fully recyclable options.

Ethanol

Laboratories are currently required to source molecular biology grade (200 proof) ethanol for the preparation of sampling kits. The term ‘molecular biology grade’, designates that a product has been tested for specific contaminants such as RNase, DNase and Proteases, and has been specifically purified and assayed for use in molecular genetic applications. The fact that we are adding sampled environmental water to the ethanol (a sample full of nucleases), renders using this grade of ethanol largely redundant. Once added to the preservative solution, the degradation control DNA would be expected to form insoluble aggregates that would be protected from the action of nucleases that would normally be expected to degrade solubilised DNA. By removing the need to use molecular biology grade ethanol to alternatives of equivalent purity (but not certified nuclease free) there could be reductions in cost and easier sourcing of this preservative. Whilst ethanol is used in large quantities within GCN eDNA monitoring it should be highlighted that laboratories will be disposing of waste solvent via a commercial collection where it is recycled (discussed further in Task 3).

Sampling protocol

Between 2014-2020 the RSK ADAS sampling kit would include a printed paper version of the in-field collection protocol. In the last couple of years RSK ADAS have omitted these from the sampling packs to reduce paper waste, we work with several repeat clients and organisations who are familiar with the sampling techniques, and for others there are PDF versions available via our website and web ordering system. In our experience, we do receive a limited number of phone calls throughout the sampling season, from ecologists asking how to take the sample correctly, and have received samples sent back for analysis that have been sampled incorrectly, with mistakes ranging from: incorrect water volumes added to each tube; to the addition of water to 1 tube of the 6. This highlights that there is a need for some re-training opportunities, and this would be especially relevant if there were to be any change to the sampling protocol.

In-Laboratory recycling schemes

As well as encouraging in-field recycling (ecologists recycling plastics and packaging through domestic recycling) and a combined effort to move towards more sustainable resourcing, plastic waste in the field almost pales into insignificance when compared with the plasticware that is used within the laboratory. Recently however several laboratory recycling schemes have started to be offered by plasticware suppliers. In a strive to be more sustainable, suppliers are becoming aware of the importance to reduce plastics and packaging, and so are providing re-fill options for example pipette tips and are re-packaging/re-branding items using more sustainable and recyclable materials. Two example schemes that we are aware of are the Appcycle scheme from Appleton Woods, and the TipOne from Starlab:

The Appcycle scheme provides laboratories with a waste recycling box. They have the capability to recycle gloves, face masks and pipette tip boxes from any manufacturer. Recycling is carried out within the UK, with consumables being made into other useful items. Collection of full bins is made straight from the laboratory.

Starlabs (used by RSK ADAS): pledges the continued repackaging of products supplied by them, to more sustainable materials, effort is focussed on the reduction of single-use plastics. The Starlab TipOne system is made from 100% recyclable polypropylene, and the company also offer a free, easy to use recycling collection service. Starlab tip boxes have a recycling logo printed on them enabling us to add them to our general recycling bins.

Use by dates for kits

Assessment of the stability of sampling kits is made through the addition of a known concentration of an appropriate synthetic DNA marker, known as a ‘degradation control’. The degradation control could be more accurately described as a ‘recovery’ control as it is a measure of both any DNA degradation and the overall effectiveness of DNA recovery.

Service providers have been allowed to provide their own DNA marker, which should have no analogue in the natural world, so can be clearly differentiated from all DNA sampled in-field. During qPCR, the production of an amplified DNA product (a positive PCR reaction) is measured by the detection of fluorescence above background levels. This point is known as the cycle threshold (Ct) or Ct value. The cycle at which this Ct is achieved is related to the amount of target DNA that is present in the sample. Knowing where the Ct of a control target DNA should be, and deviation from this to a later PCR cycle would be indicative of either DNA degradation during storage or poor DNA recovery often associated with samples containing high amounts of sediment. It is the stability of this DNA marker over time that governs the storage/shelf-life of the unused sampling kit. The technical advice note currently states that 'kits should be used within around 2 weeks of receipt', this is now out of date and requires re-wording. In 2017, laboratory test data provided by RSK ADAS and other service providers which demonstrated that the shelf life of an eDNA kit could be extended to encompass a whole GCN season was shared with Natural England prior to the large-scale district licencing projects of 2018 and 2019. Allowing a kit to last a full GCN eDNA sampling season was a pragmatic approach to addressing the large numbers of kits that needed assembling. Studies showed that kits subjected to varying temperature conditions and storage duration (6, 12 and 24 months), showed 'within limit' Ct values after DNA extraction and qPCR amplification. This means that the true useful shelf life of eDNA sampling kits is far longer than currently stated. In 2018, it was agreed with Natural England that kits should be used within 3 months rather than two weeks although this has never been amended in the technical advice note. When asked, 42% of the questionnaire responders were unaware of the kits having this extended shelf-life. Relaxing the shelf-life limits to even longer periods would further cut wastage of unused kits. We suggest that the onus should be on the service provider to ensure that degradation control DNA within kits that have been stored for extended periods of time should be within the acceptable margins before they can be supplied to clients.

Ecologists were asked 'what do you do with unused kits?' Common responses were, kits would either be used for training purposes, utilised on another job, or returned to the supplier. Other responses include 'disposed of the kits', 'bin' and 'they just get left piling up in the office'. RSK ADAS request that any unused kits can be returned to us for re-distribution if there is still scope to do so within the GCN survey season. We also encourage the return of any unused kits after the sampling season (some of which are used for our own research purposes) also ensuring the correct disposal of the kit preservative. Clarity over any flexibility in the shelf-life of sampling kits would help both suppliers and their clients and reduce wastage.

Extension of the current sampling season

The GCN eDNA sampling season currently falls between mid-April and the end of June coinciding with the newt breeding season. Sampling outside of this time frame is termed 'out of season'. Results obtained out of season are not usually accepted for GCN licensing purposes by Natural England. There is limited data on the year-round detection of GCN

within water bodies throughout the year. The lifecycle of the GCN involves both terrestrial and aquatic phases where the animals may spend periods of time from March to June within waterbodies to breed and then are largely land based for the rest of the year. It is thought however that juveniles may well spend longer periods within the waterbodies and these and adults can overwinter in ponds. Whilst out of season timepoints may preclude conventional survey effort (other than juveniles the GCN are not thought to be present in large numbers), there may be smaller concentrations of eDNA that could be detected out of season. Two published studies and additional data (in preparation for publication) from NatureMetrics have sought to address this. Rees et al. (2017) looked at two ponds from the East of England, with well characterised GCN populations, by both eDNA and by conventional ecological survey for 12 months during 2014/15. Visual observations confirmed GCN activity until August but no later and they did not reappear until February. However, GCN eDNA detection in water samples demonstrated the presence of GCN eDNA throughout the year but PCR scores (number of positive reactions out of 12 replicates) were low from September to January, suggesting very low populations being present. This data might suggest that eDNA survey could be reliably expanded to encompass the months February to August. Buxton et al. (2018b) published the results of a larger study looking at a larger number of ponds in the South-east of England. This study compared the detection rate of GCN eDNA in ponds with known GCN populations in both the water and in sediments. The presented data was condensed into sampling areas and displayed as a detection probability. The authors likewise report, low detection probabilities in samples taken from the autumn and winter sampling points and suggest that they would 'not recommend using eDNA for year-round sampling without further refinement and testing of the methods'. The data provided by NatureMetrics with Atkins and HS2 Ltd also showed that GCN eDNA was detectable outside of the current survey season in July both when using ethanol precipitation or filtration for eDNA capture. From the limited data available (and on data acquired from waterbodies in the warmer parts of England), the survey season could likely be extended to late summer, and possibly brought forward from its start point in mid-April. The start date of the season is a variable that could be explored in the future, it could be that surveys may be allowed prior to the current date if the temperature was above a certain value for a number of days which may remove variability in geographic location.

Very low amounts of GCN eDNA may be being detected during the winter months but is unlikely to be able to be backed up by survey and will likely be near the detection limits of the current assay. Increased volumes of water for analysis perhaps sampled by filtration may overcome this, but where in the UK this will be applicable would need to be the subject of further research. A number of ponds with a range of eDNA scores during the survey season would need to be tested to show that low scoring water bodies can still be reliably detected outside the survey season as these are more likely to contain smaller or transient populations of great crested newt. Similar analyses of additional ponds throughout the UK are required to demonstrate how applicable these observations are to the rest of the UK. In addition, analyses of samples taken over several seasons are required to demonstrate the reproducibility of this eDNA detection across years, which may present distinct breeding conditions for great crested newt. RSK ADAS as a service

provider do offer testing for our clients out of season with the understanding that these data cannot be used for GCN licence application. It is understood that in several instances these out of season (late summer) tests are being used to get advanced notice on testing the same ponds for the next season.

In our questionnaire, a 2-part question was posed: ‘Should there be flexibility in the start and end of the eDNA season?’ and ‘If so, what factors should feed into this?’. 65% of responders gave a non-committal ‘maybe’, while 22% said yes and 13% no. This suggests a reluctance or lack of confidence to give a definitive answer without being presented with any data defining any benefits or negative aspects to a season extension. Common points raised included the significance of the ability to draw-on local knowledge and to gain easy access to site information from previous years surveys. Weather and changes in the climate were mentioned, but would mean a year on year, or month on month GCN season assessment, which would be hard to implement and add confusion to the sampling side of GCN detection. Southerly regions of the UK where the weather can be warmer earlier on in the year, could maybe start sampling earlier. Some ecologists noted that they could detect GCN presence from March and later into July. Whilst many responders provided a list of important variables for consideration before electing a change to the eDNA season, some used the platform to alert us to potential pitfalls. ‘Is it not better to have the simplicity and consistency of having fixed season dates?’, ‘Could proposing an earlier start to the season in southern regions create a confusion about when is the right time to survey?’ ‘‘Wiggle room’ on dates may not be helpful from a client perspective and could cause problems with validity of the tests for clients seeking planning applications.’

Sample quality

Samples that are returned to the RSK ADAS laboratory are visually classified as: good; low; medium; and high sedimentation; and/or presence of white precipitate; or algae. This data is collected to provide additional interpretation of GCN results. There is a positive correlation observed between increased levels of sediment, the presence of white precipitate or presence of algae, and indeterminate/inconclusive results. These effects appear more marked as the season progresses as ponds become more difficult to sample due to algal growth or ponds drying out making it more difficult to obtain a clean sample. Ecologists were asked: ‘Are you aware of how sediment and/or algae can affect our ability to generate results?’ 39% indicated that they were unaware, again highlighting some retraining requirements and possible additional emphasis of this to be written into the technical advice note. A potential solution that we have used at RSK ADAS on other projects when faced with water samples that have high levels of particulate matter is to pass the water through a 100µm pre-filter, which provides a small enough pore-size to remove large particulate debris but permitting passage of eDNA. This can mean that samples that would likely return an indeterminate result, could offer a definitive GCN result. 94% of questionnaire responders would be prepared to pass such samples through a fine mesh filter before taking the final sample. These simple pre-filters are cheap, easy to source and are recyclable/compostable.

The technical advice note states that the water column should be gently stirred prior to water collection. The questionnaire asked, ‘Do you routinely stir the water column prior to sampling as stated in the technical advice note?’ 90% of responders said that they did and 44% of these noted that the quality of the water sample decreased on stirring of the water column. It is likely that the surveyors are stirring up the sediment during this process therefore we suggest that the technical advice note further emphasises that it is important to avoid collecting sediment.

This methodology can be used for ponds up to 1ha, but for ponds that are particularly small (<5% 1ha) taking 20 samples encourages surveyors to take water from suboptimal areas where decaying vegetation/sediment cannot be avoided and may lead to poor sample quality. We suggest that the technical advice note allows for flexibility in sampling, particularly from smaller water bodies to allow fewer samples to be taken.

Another solution to decreasing the amount of sample debris and improving sample quality, is to leave the water sample to settle for 10-20 minutes after collection before transfer to the sub-sampling tubes. When ecologists were asked if they would be prepared to do this there was a mixed response: 56% of responders agreed or strongly agreed that they would be prepared to do this, whilst 33% disagreed and 10% were indifferent to the suggestion. Again, a greater awareness of the importance of obtaining a good quality sample for laboratory processing would be helpful in ensuring that samples of the highest quality were returned to the service providers, even though it would increase the time taken to sample from some of these areas. A good quality sample is far more likely to give an uninhibited result and is much easier for the service providers to work with.

A further water sample quality issue is that of the presence of a white precipitate which forms gradually after sample collection. The presence of this white precipitate makes it incredibly difficult for laboratories to extract any eDNA from the sample. As with samples of high sedimentation, the presence of a white precipitate makes it more likely that an indeterminate result would be recorded. For an ecologist who may be sampling several ponds at a site in one of these areas, there is a high chance of all samples returning an indeterminate result. RSK ADAS have tested several samples that had given a white precipitate on reaction with the ethanol preservative. Water chemistry profiling of these samples showed elevated levels of dissolved calcium ions when compared to our control samples that had not produced such precipitates (Appendix 4). We suspect that in areas where the local geology leads to high levels of dissolved calcium in the water, the calcium ions can react with the preservative creating a white precipitate, which is often only visible a few hours after sampling. A potential solution to this issue could be to include a simple ‘dipstick’ to the sampling pack to test the water chemistry. The ecologist can pre-test the water source to see if calcium levels fall within a range suitable for ethanol-based sample collection. This pre-test could be a cost-effective, pre-screen of the water quality especially for areas where hard water is suspected. Such dipstick tests for testing the hardness of the water could cost below 10p a test and would require minimal training for use. To date whilst we have determined that dissolved Ca^{2+} salts are the likely cause of these poor samples we have not trialled the concurrent use of such dipsticks as a water pre-screen and offer this as a possible research suggestion. In the instances where water may be

unsuitable for collection using ethanol precipitation a filtration method could be used as an alternative (see Question 1 for where the advantages/disadvantages of filter usage are discussed further). This issue is not described in the technical advice note yet we estimate that every season this accounts for around 5% of all samples returned. We therefore posed the question: 'Are you aware that certain types of water chemistry (likely high calcium content) can react with the preservative and interfere with our ability to generate results?' 73% of responders were unaware that this could be a problem therefore we suggest that this is raised within the technical advice note, along with these possible mitigation steps suggested above.

Summary and Recommendations

Task 2 focused on the methodology associated with the water sampling, an area for flexibility that would bring benefits in terms of sustainability and costs. There was an overwhelming requirement made by ecologists to look at reducing the extent of plastic use and packaging within sampling packs, as well as sourcing more readily recyclable items. Being granted the flexibility to not have to source 'DNA free'/ sterile consumables, would open opportunities for providers, by making more products available for consideration. This could ultimately see a reduction in plastic use and packaging that is currently called for. If consumables are not required to be supplied individually wrapped or go through extra testing to be certified as DNase and RNase free, then the provider could also see a reduction in costs although this may be negated by recent price rises. Under the current methodology the flexibility to use non-molecular grade ethanol in the precipitation could simplify procurement and reduce costs. Our reasoning is that as soon as water samples are added to the kits, high concentrations of nucleases are inevitably added to the tube.

There is evidence in peer reviewed literature of high detection rates of GCN eDNA until late summer (at least in the South of England), how this compares to the rest of England and Wales is not known and would need to be further investigated. The seasonality of the GCN eDNA testing season is widely accepted by ecologists and there appears little appetite to expand these dates.

Some points for consideration are highlighted below:

- Service providers could be cutting down on excess packaging with support from Natural England by removal of stipulation for sterile/DNA free.
- Service providers should strive to make as much of the sampling kit recyclable or compostable as possible and supply their clients with this information.
- Service providers should strive to recycle more laboratory waste as laboratory recycling schemes become more widely available.
- Natural England need to amend the protocol to use of eDNA sampling kit within a season (currently a two-week shelf-life) or decide whether service providers should be given the flexibility to use kits from previous years if they can satisfy themselves that batches show no appreciable degradation of control before shipping to clients.

- Although there is some evidence to suggest that GCN can be detected year-round, the rate of positive detections becomes lower in out of season months in limited numbers of samples from a limited number of areas in the south of England, there also seems to be little appetite for extending the survey season amongst ecologists. On the basis of current evidence, we do not recommend a change to the length of the survey season.
- There needs to be more emphasis on water sample quality (sediment/algae etc.) either by further training or amendment to the technical advice note.
- Issues to do with Ca^{2+} rich waters could be addressed by both wider training as to awareness, water quality testing and in these instances filtration-based methods could be adopted.
- All interested parties and end-users should be made aware of any changes made to the sampling methodology and there may be a requirement for further training of Ecologists especially if filtration is to be adopted.

Task 3. Evaluate the laboratory procedures specified in the current protocol (WC1067) in light of developments since 2014; and recommend any changes to the current protocols supported by relevant evidence (this could include different potential changes to the same part of the protocol)

Quality assurance and quality control

Quality assurance and quality control will always be of the utmost importance for all laboratories wishing to provide a GCN detection service. ‘Section 2. Quality assurance and quality control’ of the technical advice note, expresses a requirement for a certain level of laboratory accreditation for each provider of GCN-based services, with equivalence to ISO/IEC 17025 standards. This is a common requirement requested by regulating authorities worldwide for laboratories providing standard tests and calibration services. As well as laboratories attaining an assured level of facility accreditation, there is also a requirement to be thoroughly proficient in the GCN detection methodology. We draw attention to a required update to section 2.1 of reference document, regarding the potential need for proficiency testing. The 2014 technical advice note currently states; ‘Ultimately it may be necessary to develop a proficiency testing scheme for eDNA analysis to enable the identification of laboratories certified as achieving the appropriate level of proficiency with the eDNA methods.’ This will now require amendment to reflect the current proficiency testing scheme and we suggest the following text ‘All GCN eDNA service providers must take part in the annual proficiency testing run by FAPAS® for their eDNA results to be accepted by Natural England. The onus is on users of the service to ensure that their service provider has taken part in the scheme and that they are happy with the results achieved.’

Proficiency Testing

The proficiency testing scheme, specific for GCN eDNA detection from water sources, has been in place since 2017, with laboratories being assessed annually between late January and early February (pre-GCN season). Proficiency testing schemes are important in maintaining high-quality service provision for clients. Being the first, and currently the only, eDNA-based proficiency testing scheme available, means that the GCN proficiency testing system can be used as used as a comparative standard for all future proficiency testing accreditations bought in for detecting other target species (Trujillo-Gonzalez et. al. 2021).

Providers are required to send their own ethanol preservation/precipitation kits (duplicate set of 10 samples) to accredited proficiency testing provider for the food and water industry, FAPAS® (Fera Science Ltd). Returned sampling kits are set-up by FAPAS® and run as blind-test samples, with the aim of assessing a laboratory's performance in detecting the presence or absence of GCN DNA at different eDNA concentration ranges (low, medium, high) that falls within, the working sensitivity of the assay. The scheme scrutinises the processing capabilities of each laboratory, testing systems that are in place to prevent sample cross-contamination as well as being able to identify the presence of PCR inhibitors that may result in a false negative result. Results are tabulated by FAPAS®, with each laboratory given a reference ID to maintain anonymity. Each laboratory is assessed as satisfactory against each sample for correct detection of GCN DNA (positive samples), correct absence of GCN DNA (negative samples) or inconclusive detection (for inhibited samples). It is not a requirement for laboratories to declare proficiency testing results although many service providers choose to do so as clients are aware that their service providers need to take part in the annual proficiency testing scheme for their results to be accepted by Natural England and they want to ensure that they are getting a high-quality service. However, since 2017 there has only been one year (2021) where all laboratories taking the proficiency test achieved 100% sample identification (Figure 1). In all other years, the overall combined satisfactory performance ranges 84-99%. The performance for individual samples ranges 63-100%. Some of this can be explained by laboratories missing low eDNA concentrations, in other instances there is evidence of PCR contamination or sample misidentification. It is concerning that in some years, and with a very low number of samples, some laboratories have generated false positive data where a sample that was known to be negative and not contain any GCN DNA was found to be positive for GCN DNA. eDNA service providers that make an error in a proficiency test are responsible for their own investigation and implementing preventative actions. Proficiency test sample kits are provided in duplicate, so that one set can be used for the proficiency test and the second set to be used for investigation and corrective actions. Currently there is no demand from the eDNA service providers for more frequent than an annual proficiency test. Given that some service providers are not achieving 100% satisfactory proficiency testing results in any one year using the existing standard methodology should any change to the protocol be permitted? There has never been a recommended standard at which service providers are deemed competent, the scheme is run so that providers can assess their own competency and adjust their practices if they are being seen to fall short of requirements. Service providers that are not meeting a minimal standard should be given the opportunity to repeat the proficiency test once any necessary improvements have been implemented. There is a need for further clarity on this. (Fapas® proficiency test data used with permission of Fera Science Ltd)

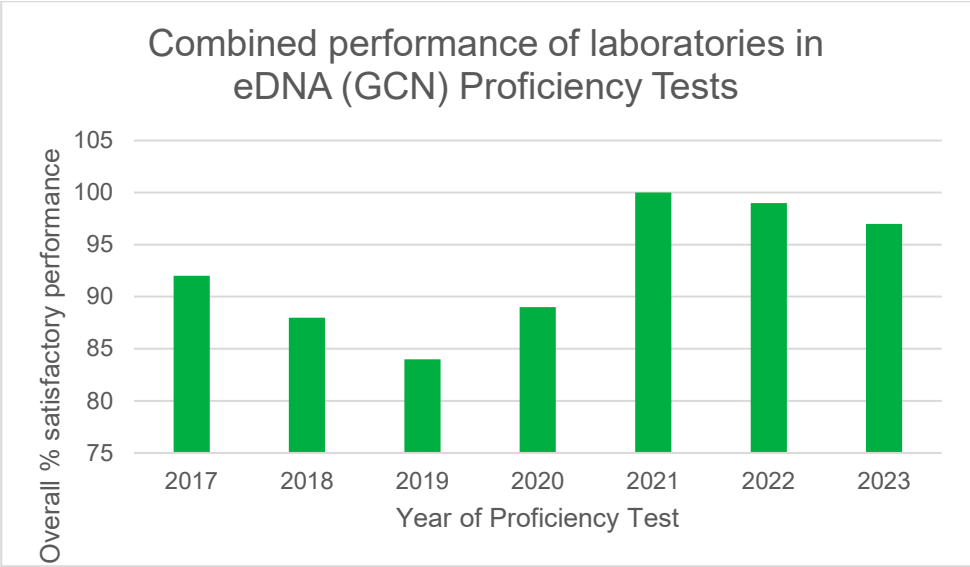

Figure 1. Percentage of laboratories passing the proficiency test each year with 100% score (Fapas® proficiency test data used with permission of Fera Science Ltd)

Technical advice note WC1067 currently states that 'the main principles of the laboratory set-up should be following (PHE, 2013)'. We draw attention to the latest edition of the Public Health England reference guide (PHE, 2018) and suggest an update should be made to this more recent reference.

According to the technical advice note 'Biggs et al. (2014) achieved a Limit of Quantification (LOQ) of 3 x 10^{-3} ng/L: at present there is no evidence that great crested newt eDNA can be quantified with precision and accuracy below this level.' A limit of quantification of 3 x 10^{-3} ng/L, would mean each laboratory was capable of consistently detecting GCN DNA to a concentration sensitivity of 3 x 10^{-9} ng/25µL reaction. However, the paper later published from the study (Biggs et al. 2015) when describing the method used to calculate the LOQ and LOD sates that 'a dilution series of a known amount of great crested newt DNA, ranging from 1 x 10^{-1} ng/µL to 1 x 10^{-10} ng/µL' was used. If this was the case the amount of DNA in each of the 12 replicates per dilution would range from 3 x 10^{-1} ng/25µL reaction to 3 x 10^{-10} ng/25µL reaction. The paper goes on to state that the LOQ in the study was 3 x 10^{-3} ng (per 25µL although not stated in the paper) with great crested newt DNA still detectable at a concentration of 3 x 10^{-9} ng (per 25µL although not stated in the paper) which was set as the limit of detection (LOD). We suggest that the terms LOQ and LOD have been confused and that the LOQ stated in the technical advice note should be the LOD. We suggest that in order to be able to compare the true interlaboratory sensitivities of the GCN PCR, during the proficiency testing, Fapas® provides each laboratory with the same GCN starting material to allow each participating laboratory to produce a standard curve from which calculations of LOD and LOQ can be made.

Laboratory protocol

Each section of the laboratory protocol will be considered in the sections below.

Sample processing

Sampling kits presently arrive back at the laboratory as 6 x 50mL sub-sampling tubes, each containing molecular biology grade ethanol, sodium acetate, a synthetic DNA control and 15mL of environmental water sample. As described in question one, eDNA is precipitated from solution in the presence of sodium acetate and is pelleted during centrifugation at 14,000 x g for 30 mins at 6°C. This 14,000xg centrifugation speed contrasted with that of Ficetola et al. (2008) and Thomsen et al. (2012), the latter being the group that published the primers and probes used in the technical advice note. These groups both used a centrifugation speed of 5,000xg and the methods in the technical advice note were one of the first to use this higher centrifugation speed (Biggs et al. 2014; Biggs et al 2015). To compare these two centrifugation speeds RSK ADAS collected replicate samples from 21 water bodies and subjected one set of samples to centrifugation at 14,000xg (as per the technical advice note) and the other at 5,000xg (as per Thomsen et al. 2012). All other steps were performed as per the technical advice note with the results showing that all the samples were positive at both the centrifugation speeds and there was little different in the PCR scores and average Ct values (Appendix 5). The use of the lower centrifugation speed would allow lower specification (often cheaper) centrifuges to be used as they would not need to run at such a high speed. This would also be the case for the 50mL centrifuge tubes as they would not need to withstand such high centrifugation speeds.

After the centrifugation step the supernatant (Ethanol/sodium acetate and water sample) is discarded into suitable containers which should be stored in an appropriate flammables' cabinet, whilst awaiting disposal/recycling in strict adherence to the UKs hazardous waste regulations, by specialist ethanol waste contractors. The route for disposal of ethanol is detailed in the chemical material safety data sheets but not currently referenced in the technical advice note. Given the volumes involved we suggest that this should be added to ensure that all service providers are reminded of regulations.

Attention is drawn to Question 1, where the ethanol precipitation kits and alternative filtration technologies for DNA capture are discussed. A potential move to a filtration methodology (or part move to allow both ethanol precipitation and filtration to be used) would negate the need for disposal of hundreds of litres of ethanol per laboratory each GCN season. A move to filter kits would also introduce flexibility in couriering for dispatch and return of kits as ADR compliant couriers would no longer be required for the transport of ethanol (dangerous goods in limited quantity). Providers are currently reliant on couriers that will transport the ethanol kits who charge a premium for the service and require that staff dispatching the kits have a suitable level of ADR training to ensure that the ethanol kits are packaged correctly. Courier costs for a single sample can be expensive (see Question 6) but are more reasonable when larger numbers of samples are sent (up to 25 samples per consignment).

DNA Extraction

The past decade has seen remarkable growth and interest in the use of eDNA from water sources as a tool for targeted species detection and biodiversity assessments. A principal step in the effective processing of water samples post-eDNA capture, is the extraction of eDNA. Extractions made from lentic and lotic water sources can pose a variety of potential issues, due to the complexity of each individual ecological matrix. GCN eDNA analysis is from lentic water bodies, which have the propensity to have considerable diversity in turbidity, vegetation, algae content, sediment, and for an individual pond this can change during the sampling season. This can mean that organic and inorganic PCR inhibitors can cause problems throughout the in-laboratory sample processing steps. Plant-derived substances often present in aquatic, soil, and sedimentary environments, such as humic, tannic and fulvic acids, are complex mixtures of polyphenolic compounds, well known as natural inhibitors of qPCR (Schrader et al. 2012; Kreader et al. 1996). Humic acid has been shown to interact with template DNA (Opel et al. 2010) or interfere with DNA polymerase (Sutlovic et al 2008). Adverse water chemistry, such as high levels of dissolved calcium ions (resulting in the presence of a white precipitate in ethanol sampling kits), also make it exceptionally difficult for laboratories to generate results.

Each year around 8% of eDNA samples returned to RSK ADAS are found to show inhibition in a PCR specifically testing for sample inhibition. It is therefore useful to consider how we can reduce the potential for samples to contain inhibitors and what (if any) changes to the technical advice note should be proposed to mitigate for this. The below list summarises potential changes that could be considered for laboratory methods.

1) The option to apply pre-centrifugation for high sediment samples prior to sample centrifugation – a short, low-speed centrifugation could be used to pellet sediment in samples that are considered to have a high or medium sediment load. The samples would then be transferred into fresh 50mL centrifuge tubes for continued processing.
2) The option to apply pre-spins for high sediment samples during the DNA extraction post-lysis/PK digestion, but pre-column loading, to prevent column ‘clogging’. This is currently a daily laboratory issue experienced when using the ethanol precipitation kits. A high sediment sample can add considerable time to processing the sample.
3) The Taq Polymerase of choice - The use of a single DNA polymerase (Taqman Environmental Mastermix 2.0) standardises the analysis across laboratories, which is useful but does not allow for improvements in enzyme technology- those that may be more tolerant to inhibitors for example (discussed in the PCR section below).
4) The addition of an optional inhibitor removal step via the use of a PCR inhibitor removal kit to further purify the DNA where DNA extracts are found to inhibit the PCR (discussed in the PCR section below).
5) Addition of substances to PCR (BSA, betaine, DMSO etc.) which can reduce the effect of PCR inhibitors (Kreader et al. 2012).

The use of commercial DNA extraction kits can be expensive, but the benefits to the user far outweigh use of more traditional methods of DNA extraction using organic Phenol chloroform-Isoamyl alcohol reagents. A DNA extraction kit provides a standardised set of reagents that are easier and safer to use. Reference document WC1067 currently has a requirement to use the DNeasy Blood and Tissue extraction kit (Qiagen®) for extraction of eDNA for GCN presence analysis. This kit is a popular choice with research groups needing to obtain high quality and yields of DNA from water bodies (Lear et al. 2017). The DNA extraction method using this kit is clearly described in the technical advice note WC1067 however, there are a few minor amendments which could be made listed below:

- In step 2 it would be recommended that the supernatant is transferred by laboratory personnel via pipetting from tube to tube and not, as currently written, 'poured'. This will reduce chances of cross-contamination as well as ensuring the collection of more of the supernatant from each tube as 'pouring' would result in larger loses in volume.
- In step 3 in the interest in saving on plastics, the supernatant should be retained in the sixth sub-sample 50mL tube and not transferred to a 2mL tube as further steps can be performed in the 50mL tube just as easily.
- Step 4 refers to an '11^{th} tube' which does not make sense, we suggest that the text is amended with text similar to the following 'One or more extraction blanks should be performed daily with every set of samples that are processed. The extraction blanks consist of all the DNeasy blood and tissue kit reagents used to this point and no sample. Extraction blanks are used to check for possible cross-contamination whilst processing samples and should be performed in parallel with eDNA samples.'
- We also suggest that the text referring to a 'quality of alcohol' test should be modified as it is not clear what is meant by this, we assume this is referring to a negative control test kit carried out with GCN DNA free water. We suggest that the text is replaced with text like the following 'Negative field sites should also be tested periodically throughout the season these being either out of range sites where great crested newts have been confirmed to be absent or sites within the newt's range where there is a certainty that newts are absent. Additional control samples may be added to the process depending on where it is believed contamination may be originating.'

Whilst the DNeasy blood and tissue extraction kit is a popular choice in eDNA studies, alternative extraction kits are now on the market specifically targeting eDNA studies and therefore a comparison with other commercially available kits and protocols used in the literature is necessary to investigate whether alternatives could be used.

Deiner et al. (2015); Compared extraction of eDNA from two lotic aquatic systems (lake and river), with commercial DNA extraction kits; DNeasy Blood and Tissue (Qiagen®); MO BIO's PowerWater® DNA isolation kit (MO BIO Laboratories, INC. Carlsbad, CA, USA. (now known as 'DNeasy PowerWater® kit' (Qiagen®)), and a modified phenol-chloroform-isoamyl extraction, to determine if freshwater biodiversity was affected by choice of DNA capture and method of extraction. The PowerWater® DNA Isolation Kit was developed to isolate genomic DNA with initial DNA capture by filtration technology. It boasts a patented Inhibitor Removal Technology® (IRT), which provides DNA of high quality and yield, even

from water sources with high levels of contaminants. DNA capture is via filtration or ethanol precipitation. Results demonstrate the requirement for utilising alternative protocols dependant on different biodiversity targets. DNA capture by filtration followed by extraction using the DNeasy blood and tissue extraction kit (Qiagen®) was best for studying eukaryotes, whereas DNA precipitation and extraction using the MO BIO PowerWater® kit was best for studying eubacteria as it detected more genera.

Djurhuss et al. (2017) investigated the same three methods for eDNA extraction studying the effect of each method, along with filter membrane type, on the biological composition and richness of communities across multiple trophic levels in seawater. Their data supported the use of both extraction kits for a 'one-size-fits-all' approach to monitoring marine biodiversity, whilst the third method of extraction using organic solvents was shown to not exhibit the same community diversity. The overall extraction procedure was also noted as being less time consuming to complete then the DNeasy blood and tissue kit.

Hinlo et al. (2017) studied combinations of DNA capture, preservation, and DNA extraction methodologies on the detection of a single target species from aquaria. The study applied the two most used commercial extraction kits for eDNA from water; DNeasy Blood and tissue kit (Qiagen®) and the MO Bio PowerWater kit. Data revealed that the DNeasy Blood and tissue kit outperformed the MO BIO kit, with each DNA capture and preservation method combination, in terms of efficiency and yield of DNA, apart from when DNA capture was made with a polyether sulfone (PES) filter membrane. Once again, they drew attention to the importance of choosing the correct capture, preservation, and extraction method, as it can significantly affect DNA yields.

Lear et al. (2017) summarised the approaches taken in the analysis of environmental DNA from a broad range of taxa and environmental matrices, to help identify key methods for DNA extraction, storage, amplification, and sequencing from environmental samples. The results showed that the DNeasy blood and tissue kit and the DNeasy PowerWater were the top two kits utilised for extraction of eDNA from water. When the DNA studies were focused on eDNA from a particular taxa when the taxa of target species of interest was 'amphibian', then the DNeasy Blood and Tissue kit became the method of choice.

The DNeasy blood and tissue kit has been shown to be a reliable method for eDNA extraction from diverse water sources, as is clearly indicated in its continued popularity in studies where high quality eDNA are required from lentic or lotic environments (Lear et al. 2017). It is unquestionably a 'tried and tested' method for the sensitive targeted detection of low concentrations of GCN eDNA, having been the specified method for GCN methodology. However, the DNeasy PowerWater extraction method, is a viable alternative, having shown higher DNA yields then the Blood and tissue kit when extracting from certain water matrices and certain filter membrane technologies for example PES membrane filters. Comparative studies of DNA extraction effectiveness from filters suggest that the Qiagen DNeasy PowerWater® DNA extraction kit was less likely to extract PCR inhibitors along with DNA when compared with the Qiagen DNeasy Blood and Tissue kit (Eichmiller et al. 2016). If Natural England were to introduce more flexibility in choice of DNA extraction kit, then it would be appropriate to suggest that both the DNeasy Blood

and Tissue kit and the DNeasy PowerWater kit, could be used- the Powerwater kit especially in cases of poor water quality.

qPCR of eDNA extracted samples

PCR inhibition

PCR inhibition can affect eDNA samples from any environment (Jane et al. 2015) however, the stagnant nature of pond waters mean that they are prone to build up of PCR inhibitors. PCR inhibition can cause false negatives so samples should be tested for the presence of inhibitors using amplification of internal positive controls or by spiking reactions with control DNA that will not be found in the sample (Doi et al. 2017). The technical advice note describes this latter method where a known concentration of synthetic DNA is added to each sample in the qPCR reaction. Inhibition can be shown by a complete absence of amplification, or as an increase in Ct value when compared to a non-inhibited control. An increase in Ct signifies that a sample has taken longer to amplify than expected and would indicate that there has been inhibition of the Taq enzyme and/or binding of the inhibitor to the template DNA reducing the efficiency of amplification. As there is no explanation within the technical advice note, slight levels of inhibition, where Ct values are within 2 Cts of a control, are regarded by RSK ADAS as acceptable and samples that fall outside this range are considered inhibited. We suggest that there is a need for a standardized acceptable Ct range to be added to the technical advice note.

When inhibition is detected, the technical advice note advises the dilution of the sample ‘twice; which has been assumed to mean by half (1 in 2) before running the GCN qPCR. This should be clarified in any amended document. The idea being that this dilution would negate any detrimental effects of the inhibitors. Dilution of samples and/or reducing the volume/concentration of PCR template DNA have been previously recommended (Biggs et al. 2015, Takahara et al. 2015). This is not good practice, as these methods could reduce the target DNA concentration below the limit of detection (Harper et al. 2019). There should be emphasis on the need to remove inhibitors before PCR is carried out. Some DNA extraction kits contain inhibitor removal steps (Buxton et al. 2018b, Sellers et al. 2018) and stand-alone clean up kits such as those sold by Zymo or Qiagen can be used after DNA extraction (McKee et al. 2015, Williams et al. 2016, Niemillar et al, 2017, Mosher et al. 2018). We suggest the addition of an optional inhibitor removal step (via the use of a PCR inhibitor removal kit to further purify the DNA where DNA extracts are found to inhibit the PCR) is added to the technical advice note.

Interestingly the possible analysis outcomes (positive, negative, indeterminate) are not discussed in the technical advice note. There is often confusion from ecologists on what a result means (we already add this to our results documents) so it would be useful to add a short description of these. More importantly text should be added to state what constitutes a positive detection of GCN, this is currently not included in the technical advice note but is taken from Biggs et al. (2015) which used at least one qPCR replicate in twelve showing a positive result to set the limit of detection.

Degradation

To monitor for eDNA sample degradation, sampling kits contain a known concentration of synthetic DNA marker known as the degradation control. Service providers have been given the freedom of supplying their own control, or purchasing one supplied via Spygen. We suggest a rewording of the technical advice note here, as far as we are aware Spygen have not provided this DNA marker to anyone. Currently the technical advice note does not state when DNA degradation should be tested and only mentions DNA inhibition in step 5 of the laboratory protocol. We suggest that degradation testing should be added into step 5, post-inhibition testing and prior to GCN testing. It is a requirement of the technical advice note that 'details of the marker used, expected rates of decay and actual decay rates should be published alongside eDNA results for the target species'. A question worth asking is whether a sample classed as 'degraded' by one laboratory would have the same classification when tested by another laboratory? There is no information in the technical advice note as to how to define if a sample is degraded or not and we suggest that this is clarified. Without consensus in the technical advice note, it is likely that service providers will be running this part of their assay to different levels of stringency. RSK ADAS uses a cut-off of 3 Cts from a qPCR degradation control. The qPCR degradation control is subject to the same DNA capture and extraction methodology as each of the processed samples, thus providing us with a comparable control to which we would be able to identify any degradation and/or DNA recovery. An increase of 3 Cts from the extracted qPCR control is equivalent to samples being diluted 1/10. We have tested this with a range of GCN sample positivity and confirm that these can still be amplified on the GCN qPCR (RSK ADAS in-house testing). There is room for more clarity in this area as there is scope for variation in service provider assay stringency.

GCN qPCR

The primers and probe used for GCN species-specific detection have been subject to a high level of validation over the 10 years since their initial description by Thomsen *et. al*. (2012). The GCN primers and probe allow for high sensitivity detection of GCN eDNA with species-specificity. PCR analysis of DNA from closely related species, *Triturus marmoratus* (marbled newt, a related but not UK native species), *Triturus carnifex* (Italian crested newt, invasive to UK), *Lissotriton vulgaris* (smooth newt, native to the UK), and *Lissotriton helveticus* (palmate newt, native to the UK), and other UK native frogs and toads (Rees et al. under review) all show an absence of amplification, confirming specificity to the great crested newt (Thomsen et al. 2012; Biggs et al. 2014; Rees et al. 2014). No further PCR based assay has been described for this species in the last 10 years. There is no need to redesign the primers and probe to improve test sensitivity and specificity.

Advice from reference document WC1067, is to run 'a dilution series of *T. cristatus* DNA, ranging from 10-1 ng µL-1 to 10-4 ng µL-1 (increments 10-1, 10-2, 10-3, 10-4)' on each GCN qPCR plate run to act as 'standards'. We argue that the inclusion of controls at such high 10^{-1} and 10^{-2} GCN DNA concentrations only act to increase chances of cross-contamination during PCR plate set up and offer no advantage to the significance/validity

of results. It is suggested that providing lower GCN DNA concentrations as positive controls are all that is required to prove the sensitivity and efficiency of the qPCR assay. Negative controls for qPCR would still include a DNA extraction blanks, run as replicates of 12 (same as a normal sample), and qPCR negative controls (using ultrapure water of molecular biology grade), run as replicates of four.

Current methodology for qPCR involves a reaction volume of 25µL within a 96-well plate using a TaqMan® Environmental Master Mix 2.0 (Life Technologies ®) with an AmpliTaq gold DNA polymerase, a hot-start enzyme which is inactive at room temperature. The hot-start characteristic negates problems of mis-priming and elongation at room temperature, which could result in amplification of non-specific targets. The Taq polymerase is tolerant to samples containing 'high levels' of inhibitors (see ThermoFisher product description) and supports a standard mode qPCR protocol with cycling duration of approximately two and a half hours. PCR reaction volumes vary greatly with volumes between 10 and 40µL having been used in the past (Rees et al. 2014). The reaction volume could be decreased in line with many other eDNA assays which have been developed which would reduce costs (for example: Mauvisseau et al. 2017; Reyne et al. 2021). Likewise, a 384-well plate could be used in place of a 96-well plate which would allow more samples to be run concurrently although a side-by-side comparison has not been carried out to our knowledge.

Even with the care that is taken to reduce co-purification of PCR inhibitors their complete removal is not absolute. Some extracted samples can show obvious visual indications that inhibitors may be present for example a brown/green coloured eluate after DNA extraction. Uchii et al. (2019) evaluated inhibition resistance of 6 commercial PCR master mixes; TaqMan Gene Expression Master mix (GMM), TaqMan Environmental master mix 2.0 (EMM); TaqMan fast advanced master mix (FMM); TaqPath qPCR master mix CG (TMM); KAPA3G Plant PCR kit (K3G); Probe qPCR mix (PQM), to three pure plant-derived inhibitors (humic acid, fulvic acid and tannic acid), and to multiple natural inhibitors (using in-field DNA samples), for detection and quantification of environmental DNA using qPCR. Polymerases under scrutiny supported the use of 'fast mode' qPCR cycling e.g., 1 s for denaturation and 20 s for annealing and extension, and/or 'standard mode' qPCR cycling for example 15s for denaturation and 1 min for annealing and extension. The appeal of using fast-mode qPCR is the potential reduction in PCR run times. GMM and FMM showed lower resistance to humic and tannic acids compared to the other master mixes and were left out from tests against multiple natural inhibitors. None of the master mixes exhibited inclusive 'best resistance' to all 'pure' inhibitory substances when running a standard mode qPCR, however the fast mode real-time master mix TMM, showed comparatively higher resistance to all three pure inhibitors, thus claiming a rank as 'first choice' master mix for resistance against these inhibitors. Performance of each master mix was also tested in the presence of multiple natural inhibitors. No significant differences were noted in Ct values for EMM, TMM and K3G, illustrating no inhibition detected with most field samples, although the Ct values for TMM samples appeared consistently smaller. However, TMM showed complete inhibition in 2 samples which were not seen when analysed by EMM and K3G and similarly, inhibition was noted in particular field

samples for EMM and K3G when no clear inhibition was observed for the other reagents. These results indicated that no one mastermix tested possessed an overall performance advantage to combined inhibitory effects caused by multiple inhibitors.

Sylphium Molecular Ecology market an eTaq qPCR master mix. This is a hot start polymerase, whose performance has been validated alongside the TaqMan Environmental master mix 2.0. Their in-house study looked at inhibition resistance of both master mixes to increasing concentrations of humic acid. This eTaq shows around twice the tolerance to humic acids than Taqman environmental mastermix. Also of note is Sylphium's GCN detection kit, which comes complete with primers, probe, mastermix and control DNA. It is not clear from available information if the primers and probe are the same as those currently used within the assay described in the technical advice note but the assay is tailored to be 100% species-specific to GCN (Sylphium SYL114).

There is potential to make use of alternative master mixes that appear equivalent to the current environmental mastermix, although there has been no validation with regards to GCN eDNA samples on the market. Of particular interest are those with polymerases requiring fast-mode qPCR cycling conditions as well as a high resistance to inhibitors, as demonstrated by TaqPath qPCR master mix CG (TMM). Utilising a fast-mode polymerase master mix could reduce the time taken for PCR cycle completion, which currently takes two and a half hours. This would increase the number of samples that could be analysed within a set timeframe. Of course, this has not currently been validated in comparative experiments with the currently used polymerase using pond waters and GCN as target species detected. Any changes to the methodology should be required to demonstrate equivalence with the current methodology through the proficiency testing scheme run by FAPAS®. With any flexibility, it is more important to maintain quality assurances and standards, to maintain the 'laboratory to laboratory' uniformity of results. Detection sensitivities should still be comparable to in-field population surveys and should not detect such low concentrations of eDNA that the species of interest is no longer in the water body under scrutiny.

Knowing and wholly appreciating the limitations of any survey method is essential to a service provider. A failure to fully consider sampling efficiency, sampling bias or other methodological limitations can lead to erroneous conclusions (Buxton et al. 2022). Buxton et al. applied a multiscale occupancy model to approximate pond GCN habitation rates as well as estimate potential false error rates that may occur or be introduced during in-field (stage 1) and in-lab (stage 2) eDNA protocols, which would adversely influence a laboratories ability at obtaining a 'true' positive or negative result. Conclusions led to a warning to the eDNA community advising against consigning an amplification threshold of 1 positive qPCR for assigning GCN occupancy as is the case in the technical advice note. It was reasoned 'unwise' and that an increase in the threshold beyond a 1/12 or 2/12 should be sought to reduce the estimated stage 2 (in-lab) false positive error rates. However, this modeling has not considered the data generated from the extraction of true negative control samples, which are extracted alongside samples of unknowns every day. Over the last four GCN seasons from 2019 – 2022, the RSK ADAS laboratories have run ~ 430 extraction blanks of 12 replicates each, equating to 5160 PCR replicates. If

we follow conclusions made by Buxton et al. (2022), then we would have expected to see approximately 103 false positives from the 5160 replicates. There were no false positives observed in these extraction controls as well as no false positives recorded in the 4 x replicates of negative (Molecular biology grade water) qPCR controls that have been run on every GCN qPCR plate over the four seasons (totaling 6,804 reactions). If '0' false positives have been acknowledged in our extraction and qPCR control samples, then any 1/12 and 2/12 scores we have seen should be true positives. With the provision of a highly regarded GCN proficiency testing scheme available, laboratories can confidently detect low DNA concentrations to this level of sensitivity where a 'true' 1/12 or 2/12 result is achieved and confirmed by FAPAS®. False negatives are far more difficult to account for, as samples can be inhibited. A good indication that laboratories are obtaining a required level of assay sensitivity is recognized by the fact that eDNA based GCN occupancy closely compares/mirrors that achieved with in-field population surveys by experienced ecologists (Rees et al 2014, Biggs et al 2015).

Buxton et al. also draw attention to the high number of replicates currently used to confirm GCN presence (12 reps), along with the high number of qPCR cycles (55), both of which are suggested to have contributed to elevating the in-lab false positive error rates to an estimated 2% per qPCR replicate. We agree with the authors that the assay has a very high number of qPCR cycles. A greater number of PCR cycles are recommended for samples with low DNA/high inhibitor concentrations (Lear et al. 2017). This high cycle number is in line with other assays published at the time, but this may be because of the methods of Ficetola et al. (2008) being duplicated early on in eDNA analysis research (Ficetola et al. 2008; Thomsen et al. 2012; Talahara et al. 2013; Dejean et al. 2012 etc.). As most positive amplifications have a Ct value of 32 to 44 (Figure 2, data from RSK ADAS 2022 GCN eDNA season) with only a small percentage of positive amplifications occurring at over a Ct of 44 (3.79%), a higher level of stringency would be to run PCR cycles to 50 rather than the current 55. Of the 29 positive amplifications with a Ct value of 50 and over, 8 were from samples that scored 1/12, two were from samples with a score of 3/12 (with all replicates having a Ct greater than 50), and 15 were from samples that scored ≥2/12. If the number of cycles was reduced to 50 this may alleviate the possibility of the occurrence of false positives suggested by Buxton et al. (2022), whilst only preventing 10 samples from resulting in a positive outcome or 2.4% of all positive samples during the 2022 GCN eDNA season, approximately the rate of false positives suggested by Buxton et al. (2022).

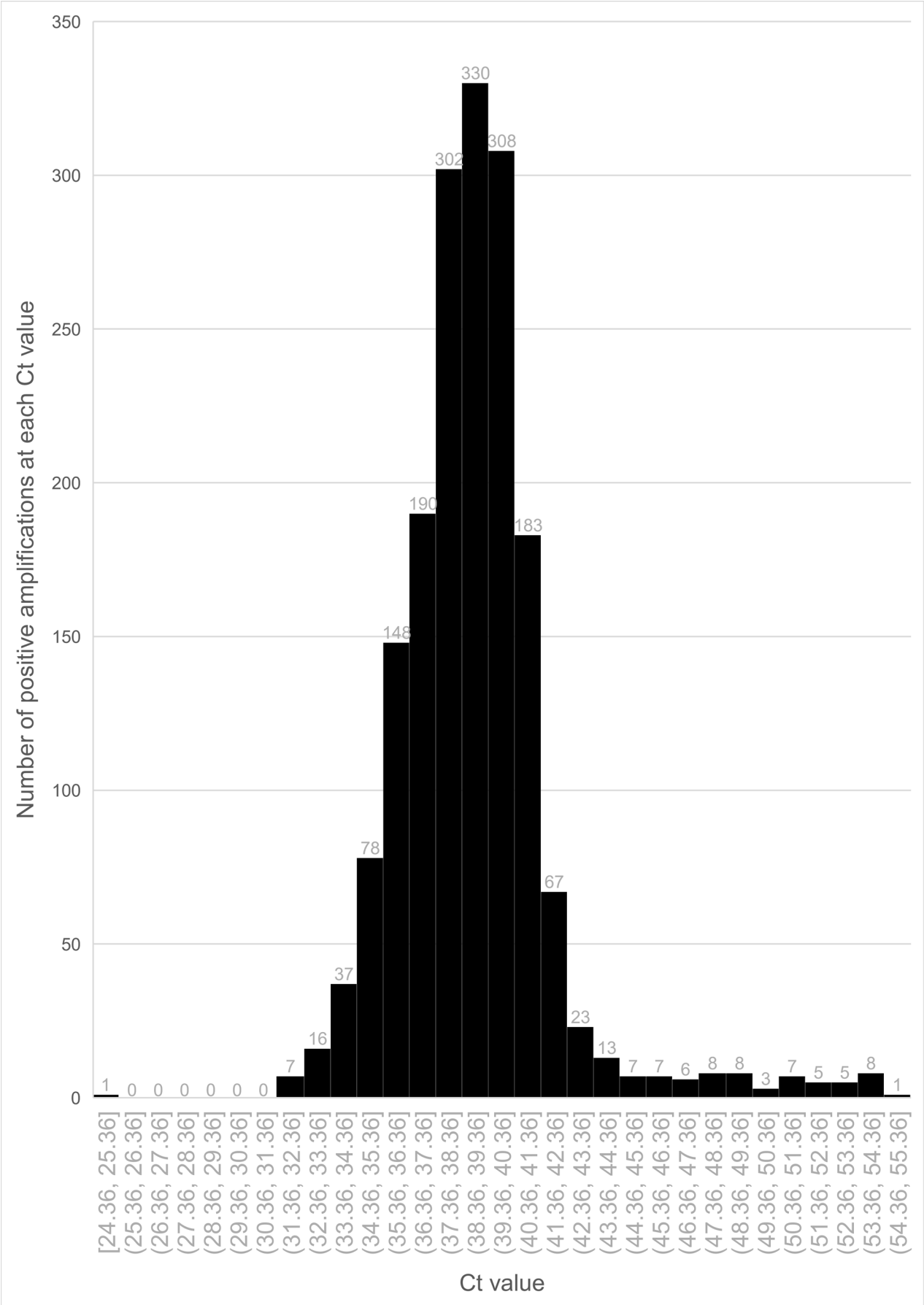

Figure 2. Histogram showing the number of positive amplifications at each Ct value for the 2022 GCN eDNA season.

Summary and Recommendations

Task 3 highlighted a potential alternative to using the current DNeasy Blood and tissue kit (Qiagen), the DNeasy PowerWater® kit (Qiagen)(Formally; MO BIO PowerWater). This extraction kit has been designed for use in the extraction of eDNA from aquatic sources containing high levels of contaminants, and is a popular choice by research groups, along with the Blood and tissue kit, for extraction of eDNA from both lentic and lotic sources. There are no other kits currently available on the market to compete with the popularity and performance of these two extraction methods.

There are several PCR master mixes whose qPCR performance showed equivalence to, or improvement on, that of the current recommended TaqMan Environmental mastermix 2.0 (ThermoFisher 4396838), when tested against common inhibitors to qPCR. We draw attention to master mixes; KAPA3G plant PCR kit (Merck KK7251) and eDNA qPCR hot start mix (Sylphium SYL1003).

Some points for consideration are highlighted below:

- Careful consideration of any changes to the laboratory protocols needs to be made keeping in mind that some service providers are not achieving good proficiency testing results year on year using the existing standard methodology.
- Currently the proficiency testing scheme is set up to detect free eDNA that is in solution and not cell associated. Filters are likely to collect cell associated material and are unlikely to detect the DNA marker currently used by FAPAS®. This would necessitate a full re-design of the FAPAS® assay.
- The LOQ stated in the technical advice note should be amended to LOD.
- Service providers should be able to make changes to the protocols if they can demonstrate equivalence through the proficiency testing scheme. These include (but are not limited to) the centrifugation speed, the DNA extraction kit, and the PCR mastermix.
- Service providers should be given the option to use a stand-alone inhibitor removal step rather than sample dilution and/or the option to use additives which aid in overcoming inhibition in the PCR reaction.
- More clarity is required for what constitutes an inhibited or degraded sample.
- We suggest that service providers are mindful not to push sensitivity of GCN detection too far beyond what is achievable by ecologists during in-field GCN pond population surveys.

Task 4. Via consultation create a list of any projects underway looking at GCN protocols and /or methodologies

To answer this question an Office 365 Forms survey was produced and sent to the following GCN eDNA service providers: Applied Genomics; Surescreen; FERA; NatureMetrics; Cellmark; and previous service provider Spygen. The questions asked are detailed in Appendix 6.

A total of 4 anonymous responses were obtained via the Office 365 Forms survey or via Natural England. To date, only two of the responders have supplied any further information to either RSK ADAS or Natural England. Only one of the responders, NatureMetrics, has performed direct comparisons between ethanol precipitation and filtration methods for GCN detection; additional information on this work has been supplied to RSK ADAS for inclusion in this report which has been included in Task 1 and Appendix 2 of this report. Relevant RSK ADAS studies are provided in below. Two responders have performed direct comparisons between ethanol precipitation and filtration methods for species other than GCN and one of these provided their published work on white clawed crayfish (Troth et al. 2020) which has been included in answer to Task 1 of this report. One of the responders, NatureMetrics, is currently investigating new technologies for GCN detection by developing an in-field, eDNA-based detection kit for GCN, but no data has been provided on this. Relevant RSK ADAS studies are again provided below. Two of the responders are engaged in GCN eDNA method development; however, again no data has been supplied to RSK ADAS.

RSK ADAS projects underway looking at GCN protocols and methodologies:

- **GCN seasonality:** Determining the scope and practicality of out of season testing for GCN on ponds with known GCN populations. GCN can be detected throughout the year. These data were generated from an innovate UK funded project that looked at whether metabarcoding approaches could be sensitive enough and appropriate for GCN detection (Rees et al. 2017).
- **LAMP assay for GCN**: Development of a LAMP assay for the specific detection of GCN eDNA. The LAMP assay is an alternative DNA amplification technology to PCR that can be used in field pond side using simple DNA extraction and analysis methods. A method has been developed that had demonstrated analytical equivalence to the current PCR assay (Rees et al 2022, manuscript under review).
- **Comparison of different eDNA filters with ethanol precipitation:** RSK ADAS have looked at comparing filtration vs ethanol precipitation for a limited number of ponds with known GCN populations. All filters tested (using the same 90mL volume of water as an ethanol precipitation) demonstrated a 5-10x increase in PCR sensitivity (estimated from by mean Ct values) of the filtration methods over the

ethanol precipitated samples, with all 3 filter types tested. Data shown in Appendix 1.

- **Water chemistry:** Early on in our commercial eDNA offer we noted a low number of samples that were very difficult to obtain eDNA results from (5% of samples in 2022). This was due to a white precipitate that had already formed in the sample tubes on receipt at the laboratory and which precluded efficient eDNA extraction. Chemical analysis of a number of these water samples demonstrated a range of dissolved calcium salts 11-20x higher than our control GCN positive pond water samples. We suggest that ethanol precipitation is unsuitable for samples that have originated from mineral rich water areas. Data shown in Appendix 4.
- **Centrifugation speed**: RSK ADAS have tested a lower centrifugation speed (5,000xg) in order to show equivalence with the standard 14,000xg used in the technical advice note. All 21 samples tested were positive at both centrifugation speeds and with similar PCR scores and average Ct values (Appendix 5).

When asked whether responders had any additional information on eDNA kit shelf life, two responses were received indicating that the performance of the degradation control was still stable after either 6 months or 3-4 years which agrees with our own findings over the last few years. Responses to the open-ended questions in the survey (questions 4, 5, and 11) are provided below.

Responses to open-ended questions within survey of ecologists:

Q4. What do you think the main advantages of ethanol precipitation or filtration have over each other?

1. Better detectability (more water sampled so more eDNA) and easier way to ship the samples for the filtration method (ethanol is considered as a dangerous goods).

2.Filtration enables the sampling of a larger volume of water which should in theory allow for more eDNA to be collected within the sample. The kits are also lighter in weight and depending on sampling strategy are more sustainable and reduce the amount of hazardous chemicals within the kit. However, with filters, if too much pressure is forced through then they can crack or become damaged. Also, if a pond has a high sediment level, they can clog up quickly, which is potentially less of an issue for the analysis with ethanol precipitation.

3.This is not a comparison that we have looked into, so we are unable to comment.

4.Higher volume of water with filtration which increases the likelihood of detection. Enclosed filters can reduce contamination risk

Q5. In your experience are both methods suitable for all pond types?

1. Yes if the membrane's surface of the filters is sufficient to standardize the filtered volume in every pond types.

2. Potentially, depends on sediment load of the site. Due to the increased volume of sample collected, using filters, is most likely more suitable for larger sites than ethanol precipitation.

3. We have no experience of using the filtration method for eDNA collection.

4. Filtration struggles in highly turbid waters to perform to full potential however still manages to sample larger water volumes than ethanol precipitation. This does not always correlate to detection though. Further investigation of our data set is required but there have been instances where EP has outperformed F at certain ponds, but I do not have information on habitat type available currently to know if there is a link.

Q11. Finally, do your kits make best use of recyclable plastics wherever possible and/or how have you reduced plastic wastage?

1.No response

2. We have worked with suppliers to cut unnecessary packing, i.e. thinner/lighter gloves. The majority of the waste reduction has happened in the laboratory - such as recycling pipette tip boxes etc.

3. Yes. Our 50mL centrifuge tubes are collected and taken away for recycling.

4. Yes. Further information can be provided.

The responses to this survey clearly show that there has not been much further investigation into GCN eDNA methodology development since 2014. However, several service providers have used filtration methods and their suggestion is that there could be benefits for GCN eDNA analysis although there is acknowledgement of water turbidity/filter clogging. Additionally, it was highlighted that water volume to be filtered should be standardised to maintain consistency across water types.

Task 5. Consider areas of the current protocol where flexibility could be allowed rather than being fixed, whilst still maintaining appropriate assurance in methods and result

Tasks 1 – 3 have evaluated the current protocols detailed in the technical advice note, and reviewed the potential advances made in eDNA detection from water sources over the last 8 years since inception of the GCN detection service. We draw attention to potential areas of the methodology in which flexibility could be introduced to help accomplish a more cost-effective, environmentally friendly and streamLined process, in addition to offering solutions to current complications seen during sample processing, such as inhibition or degradation of samples leading to an indeterminant result.

There are areas where the introduction of more flexibility could be implemented straight away, such as a change in consumables to 'DNA free'/readily recyclable materials. However, potential changes in flexibility over DNA capture method, extraction kit choice and master mix preference, have the potential to cause a far greater impact on the quality/ sensitivities/standards and uniformity at each stage of the GCN detection service. These alternative products/reagents have yet to be specifically tested for the targeted detection of GCN eDNA from a range of lentic water sources, and it would be our strong recommendation that thorough research be supported for each potential variation before any talk of flexibility be brought about. It would be most likely that flexibility would have to be granted, but with constraints in place, to maintain high standards and comparable results between laboratories. One constraint to consider is only offering any flexibility to laboratories who are achieving good results year on year within the current proficiency testing scheme. Whatever future mitigations are agreed, assurance of methods and results are essential and would be met through proficiency testing to demonstrate an analytical equivalence and robustness of any data generated by that new approach.

Recommendations that could be implemented straight away

1. The technical advice note currently states:

'Biggs et al. (2014) achieved a Limit of Quantification of 3 * 10-3 ng/L'

We suggest that the text is amended as follows:

Biggs et al. (2014; 2015) state that the LOQ was 3 x 10-3 ng (per 25µL) with great crested newt DNA still detectable at a concentration of 3 x 10-9 ng (per 25µL) which was set as the limit of detection (LOD).

2. The technical advice note currently states:

‘Ultimately it may be necessary to develop a proficiency testing scheme for eDNA analysis to enable the identification of laboratories certified as achieving the appropriate level of proficiency with the eDNA methods. At present a proficiency testing scheme for eDNA is not available because an appropriate proficiency testing methodology has not been established. Further research and development work will be needed to establish such a scheme.’

We suggest that the text is amended as follows:

A proficiency testing scheme operated by Fapas® is available for all laboratories wishing to provide a GCN eDNA testing service. Natural England will only accept eDNA results from laboratories participating in the proficiency testing scheme.

We also suggest that in order to be able to compare the true interlaboratory sensitivities of the GCN PCR, during the proficiency testing, Fapas® provides each laboratory with the same GCN starting material to allow each participating laboratory to produce a standard curve from which calculations of LOD and LOQ can be made.

3. The technical advice note currently states:

‘Kits can be stored at room temperature before use in an appropriate solvent store, consistent with Home Office regulations, and should be used within about two weeks of receipt.’

We suggest that the text is amended as follows:

Kits can be stored at room temperature before use in an appropriate solvent store, consistent with Home Office regulations, and should be used within three months of receipt. Older sampling kits can be used where the service provider can demonstrate that for batches of sampling kits (made up at the same time) the degradation control is still within acceptable limits.

4. The technical advice note currently states:

‘The field sampling equipment used by Biggs et al. (2014) has five components (Figure 2):

- A sterile 30mL ladle
- A sterile self-supporting Whirl-Pak plastic bag with 1 litre capacity
- A sterile 10mL pipette to resample the pond water
- Six sterile 50mL centrifuge tubes containing preservative (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents (Product Code: 10644795), sodium acetate and other markers)

- Two pairs of sterile gloves.'

We suggest the following flexibility is added:

The field sampling equipment used by Biggs et al. (2014) has five components (Figure 2):

- A 30mL ladle
- A self-supporting Whirl-Pak plastic bag with 1 litre capacity (or similar) or alternatively the bag containing the 30mL ladle.
- A 10mL (or similar volume) pipette to resample the pond water
- Six x 50mL centrifuge tubes containing preservative - Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents (Product Code: 10644795) or similar Absolute Ethanol (200 Proof), sodium acetate and DNA marker (degradation control).
- One pair of gloves.
- A 100µm (paint-filter) type pre-filter

5. The technical advice note currently states:

'Identify where 20 samples will be taken from the pond. The location of sub-samples should be spaced as evenly as possible around the pond margin, and if possible targeted to areas where there is vegetation which may be being used as egg laying substrate and open water areas which newts may be using for displaying.'

We suggest that the text is amended as follows:

Identify where 20 samples will be taken from the pond. Multiple samples can be taken from the same location where ponds are small. The location of sub-samples should be spaced as evenly as possible around the pond margin, and if possible targeted to areas where there is vegetation which may be being used as egg laying substrate and open water areas which newts may be using for displaying. Try to avoid areas of poor water quality (sediment/algae/pond weed/decaying vegetation).

6. The technical advice note currently states:

'Step 2. Open the sterile Whirl-Pak bag by tearing off the clear plastic strip c 1cm from the top (along the perforated line), then pulling the tabs. The bag will stand-up by itself.'

We suggest the following flexibility is added:

Step 2. Open the sterile Whirl-Pak (or alternative bag) by tearing off the clear plastic strip c 1cm from the top (along the perforated line), then pulling the tabs. The bag will stand-up by itself.

7. The technical advice note currently states:

'Step 1. Identify where 20 samples will be taken from the pond. The location of sub-samples should be spaced as evenly as possible around the pond margin, and if possible

targeted to areas where there is vegetation which may be being used as egg laying substrate and open water areas which newts may be using for displaying.

Step 2. Open the sterile Whirl-Pak bag by tearing off the clear plastic strip c 1cm from the top (along the perforated line), then pulling the tabs. The bag will stand-up by itself.

Step 3. Collect 20 samples of 30mL of pond water from around the pond (see 1 above) using the ladle (fill the ladle) and empty each sample into the Whirl-Pak bag. At the end the Whirl-Pak bag should be just under half full (600mL).'

A possible optional step could be added in between steps 1 and 2 as follows:

Pre-screening of water bodies in hard water areas for the presence of high levels of Ca^{2+} could be undertaken using a simple dip-stick test, generally referred to as 'water hardness test kits'. High levels of Ca^{2+} (over 200 mg/L in samples that have been chemically profiled) can interfere with the DNA extraction process and cause a result to be indeterminate. Use of such a pre-screen could reduce the number of samples that are returned as indeterminate.

8. The technical advice note currently states:

'Step 4. Once 20 samples have been taken, close the bag securely using the top tabs and shake the Whirl-Pak bag for 10 seconds. This mixes any DNA across the whole water sample.

Step 5. Put on a new pair of gloves to keep the next stage as uncontaminated as possible.

Step 6. Using the clear plastic pipette provided take c15mL of water from the Whirl-Pak bag and pipette into a sterile tube containing 35mL of ethanol to preserve the eDNA sample (i.e. fill tube to the 50mL mark). Close the tube ensuring the cap is tight.'

We suggest that an optional step is added between steps 4 and 6 (step 5 already having been removed due to the supply of one pair of gloves since 2020) as follows:

If the water sample appears to be very turbid or contains pond weeds/algae it can be pre-filtered through a 100µm paint filter/strainer (available from numerous suppliers) into a second bag OR the sample dipper bag to remove these larger particulates and improve sample quality (Figure 3).

Turbid water samples can also be left for 10-15 minutes to allow sediment to settle to the bottom of the bag prior to transferring into the 50mL tubes of preservative solution.

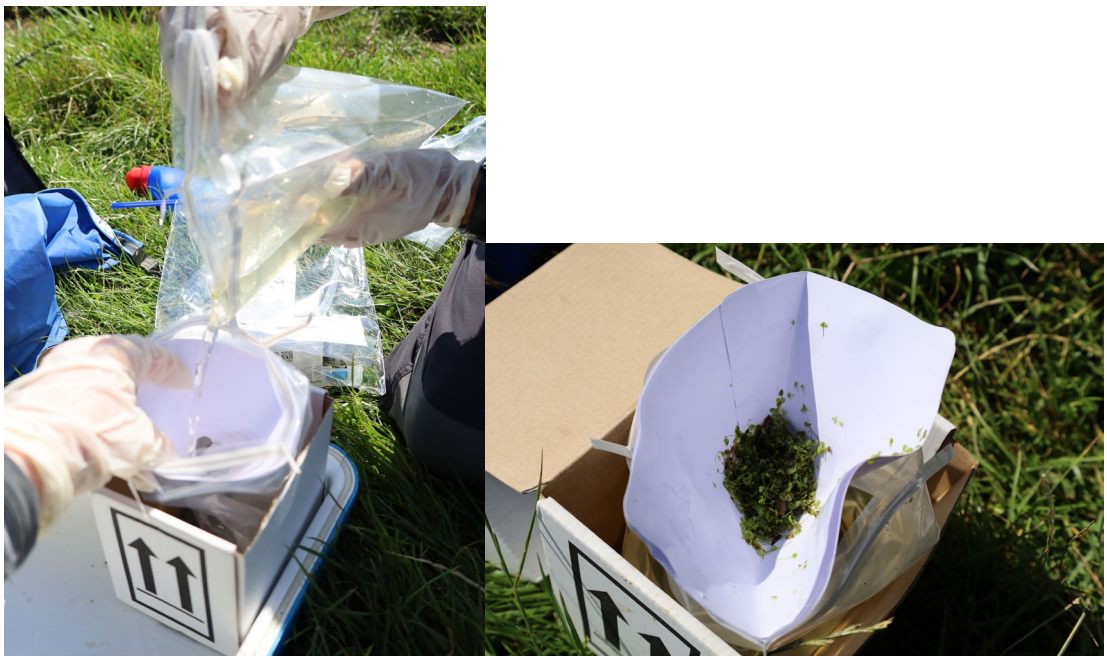

Figure 3. Demonstration of the use of a pre-filter to remove pond weed etc. © Helen Rees 2023

9. The technical advice note currently states:

'Kits can be stored at room temperature before use in an appropriate solvent store, consistent with Home Office regulations.'

We suggest that the route for safe disposal of ethanol is not currently referenced in the technical advice note but is detailed within chemical material safety data sheets. Given the volumes involved we suggest that this should be added to ensure that all service providers are reminded of regulations.

10. The technical advice note currently states:

'Step 2 360 µL of ATL Buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen®) is added to the first tube, the tube is vortexed for several minutes (time depends on degree of film accumulation on tubes) and the supernatant poured into the second tube. This operation is repeated for all the six tubes, resulting in the 6th tube containing the ATL buffer that has been vortexed sequentially in each of the six sample tubes. Vortexing is needed to remove films of DNA which become attached to the tubes at high centrifuge speeds. Flicking the tube or pipetting have not been found sufficiently vigorous to remove these films. Other kits may be suitable for this step but would need to be evaluated, perhaps as part of a proficiency testing process.'

We suggest that the text is amended to the following:

Step 2. 360 µL of ATL Buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen®) is added to the first tube, the tube is vortexed for several minutes (time depends on degree of film accumulation on tubes) and the supernatant transferred into the

second tube using a 1mL pipette. This operation is repeated for all the six tubes, resulting in the 6th tube containing the ATL buffer that has been vortexed sequentially in each of the six sample tubes. Vortexing is needed to remove films of DNA which become attached to the tubes at high centrifuge speeds. Flicking the tube or pipetting have not been found sufficiently vigorous to remove these films. Other kits may be suitable for this step but would need to be evaluated, perhaps as part of a proficiency testing process.

11. The technical advice note currently states:

'Step 3 The supernatant in the sixth tube, containing the DNA concentrated from all 6 sub- samples, is transferred to a 2 mL tube and the DNA extraction performed following the manufacturer's instructions. The DNA extraction should be performed in the room or laboratory area dedicated for degraded DNA samples.'

In the interest in saving on plastics we suggest the following:

Step 3. The supernatant in the sixth tube, containing the DNA concentrated from all 6 sub- samples, is retained in the sixth sub-sample 50 mL tube and the DNA extraction performed following the manufacturer's instructions. The DNA extraction should be performed in the room or laboratory area dedicated for degraded DNA samples.

12. The technical advice note currently states:

'The extraction control is undertaken using an 11th tube containing buffers alone and no sample (i.e. no alcohol mix and no pond water).'

We suggest that the text is amended as follows:

One or more extraction blanks should be performed daily with every set of samples that are processed. The extraction blanks consist of all the DNeasy blood and tissue kit reagents used to this point and no sample. Extraction blanks are used to check for possible cross-contamination whilst processing samples and should be performed in parallel with eDNA samples.

13. The technical advice note currently states:

'Note that the quality of the alcohol (i.e. absence of DNA contamination) is assessed with the negative controls in the field. These can be either out of range sites where great created newts are absent or sites within the newt's range where there is high certainty that newts are absent. If no negative field sites are available in a study, a different approach may be needed. In the analytical process the extraction control sample is, from Step 4 onwards, processed as a normal sample.'

We suggest that the text is amended as follows:

Negative field sites and/or tap water should also be tested periodically throughout the season. This is to test that none of the kit consumables or reagents have been

contaminated with GCN DNA during kit preparation. Additional control samples may be added to the process depending on where it is believed contamination may be originating.

14. The technical advice note currently states:

‘DNA should be extracted using the DNA Blood and Tissue kit (Qiagen®) following the manufacturer’s instructions.’

We suggest the following flexibility is added:

DNA should be extracted using the DNA Blood and Tissue kit (Qiagen) or the Powerwater kit (Qiagen) following the manufacturer’s instructions.

For samples with particularly high sediment loads a short slow-speed pre-centrifugation can be performed (2000 rpm, 1 minute) and the sample poured into fresh 50 mL centrifuge tubes for the main centrifugation at 14,000xg and/or a short pre-centrifugation can be performed prior to the sample being applied to the spin column provided within the DNA extraction kit to prevent its blockage.

15. The technical advice note currently states:

‘Step 5. DNA inhibition should be tested by adding a known amount of an artificial gene to the sample and running qPCR in duplicate. If a different than expected Ct value is observed in a least one replicate, the sample should be considered inhibited. In this instance dilute the sample twice before amplification with great crested newt primer and probes.’

We suggest the following flexibility is added:

Step 5. DNA inhibition should be tested by adding a known amount of an artificial gene to the sample and running qPCR in duplicate or by using an inhibitor screening kit. If a different than expected Ct value is observed in a least one replicate, the sample should be considered inhibited. In this instance the sample can be diluted 1 in 2 or put through an inhibitor removal kit before amplification with great crested newt primer and probes.

16. The technical advice note currently states:

‘A dilution series of *T. cristatus* DNA, ranging from 10-1 ng µL-1 to 10-4 ng µL-1 (increments 10-1, 10-2, 10-3, 10-4) and measured using a Nanodrop ND-1000 or equivalent, should be used as a qPCR standard.’

We suggest that the text is amended as follows:

Positive controls consisting of quadruplicate replicates of *T. cristatus* DNA at 10^{-3} ng μL^{-1} and 10^{-4} ng μL^{-1} and measured using a Nanodrop ND-1000 or equivalent, should be used.

17. The technical advice note currently states:

‘12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®)’

We suggest that the following flexibility is added:

12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®) or suitable alternative that has been validated and shows equivalence to current protocols.

Recommendations requiring agreement or further assessment

1. Filtration

As stated in Task 1 evidence is required to allow comparison of like for like water samples that have been collected with both ethanol and filtration to demonstrate equivalence with all water types and qualities (turbidity/Ca^{2+} content etc.).

2. Survey Season

As stated in Task 2 we suggest that a number of ponds with a range of eDNA scores during the survey season would need to be tested to show that low scoring water bodies can still be reliably detected outside the survey season as these are more likely to contain smaller or transient populations of great crested newt. Similar analyses of additional ponds throughout the UK are required to demonstrate how applicable these observations are to the rest of the UK. In addition, analyses of samples taken over several seasons are required to demonstrate the reproducibility of this eDNA detection across years, which may present distinct breeding conditions for great crested newt.

3. Spin speed

As stated in Task 3 the use of the lower centrifugation speed would allow lower specification (often cheaper) centrifuges to be used as they would not need to run at such a high speed. This would also be the case for the 50 mL centrifuge tubes as they would not need to withstand such high centrifugation speeds.

4. PCR inhibition

As stated in Task 3 we suggest that there is a need for a standardized acceptable Ct range to be added to the technical advice note.

5. Degradation testing

As stated in Task 3 we suggest that there is a need for a standardized acceptable Ct range to be added to the technical advice note.

6. Reduction in number of cycles

As stated in Task 3 we suggest that a review of data is carried out to monitor the effects of reducing the number of cycles from 55 down to 50 for the GCN qPCR in an effort to reduce potential for false positive results.

Task 6. Cost-benefit analysis

Sampling kit cost-benefits

The following tables have been compiled to illustrate what cost savings could be incurred using alternatives to the current methodology. Table 1 considers the cost associated with the supply (and return) of the current eDNA sampling kit and the likely saving that alternative kit components may bring. Costs do not include the costs of procurement; the costs associated with labour and the assembly of the sampling kits and are worked out from manufacture list pricing. These staffing costs will inevitably differ between service providers and the current market rate of £20-30 per kit is probably an underestimation of the true costs of kit assembly and provision. Shipping of samples is listed as prices compliant with ADR regulations for the shipment of the amounts of ethanol used in this methodology. Price includes shipping to ecologist and return trip to laboratory, cost savings associated with more environmentally friendly (recyclable, compostable or not individually wrapped) components are highlighted, as is an alternative non-molecular biology grade ethanol. We include the cost of ethanol disposal in these cost comparisons (Table 2).

Table 2: Comparison ethanol-based sampling kits (price per kit). The table contains examples of the consumables required other suppliers and alternative items are available. Courier costs are shown as a range of prices dependent on how many kits are placed into the box (number in brackets), couriering becomes cheaper the more kits placed into the different sized packing boxes. Total price is again shown as a range due to the different numbers of kits that can be placed into the different sized packing boxes. *This is the total price of the packing materials required i.e. bubble wrap, packing box, kit box etc.

Current ethanol-based sampling kit (per kit)				**Alternate product ethanol-based sampling kit (per kit)**			
Product	**Supplier**	**Code**	**Price (each)**	**Product**	**Supplier**	**Code**	**Price (each)**
Pair wrapped gloves	VWR	112-4555	£1.48	Pair alternate gloves	Papers-tone	HEA02758	£0.09
Biodegradable bag	Polybags	1015150BIO	£0.08	Biodegradable bag	Polybags	1015150BIO	£0.08
Whirlpack bag	Fisher	129-9887	£0.19	Whirlpack bag	Fisher	129-9887	£0.19
Pipette	Fisher	612-4515	£0.56	Pipette (LDPE)	Fisher	13449118	£0.19
Sample dipper	Fisher	15488794	£1.72	Sample dipper	Fisher	15488794	£1.72

Current ethanol-based sampling kit (per kit)				Alternate product ethanol-based sampling kit (per kit)			
Molecular biology grade Ethanol	Fisher	437435L	£5.02	Standard grade % Ethanol	VWR	83813.360	£2.90
Sodium Acetate	Merck	S7899	£0.15	Sodium Acetate	Merck	S7899	£0.15
50mL tubes	Fisher	10314131	£2.40	50mL tubes	Appleton woods	AB028	£1.62
Packaging	Various	Various	£1.91*	Packaging	Various	Various	£1.91*
Binbags for packing boxes	Polybags	Various	£0.23	Binbags for packing boxes	Polybags	Various	£0.23
Ethanol disposal	Veolia	N/A	£0.56	Ethanol disposal	Veolia	N/A	£0.56
Courier Small	DHL	Small (1-3)	£44.58 to £11.50	Courier Small	DHL	Small (1-3)	£44.58 to £11.50
Courier Med	DHL	Med (4-9)	£11.50 to £4.95	Courier Med	DHL	Med (4-9)	£11.50 to £4.95
Courier Large	DHL	Large (10-25)	£5.88 to £2.35	Courier Large	DHL	Large (10-25)	£5.88 to £2.35
Total cost per kit		**£58.87 to £16.64**		**Total cost per kit**		**£54.22 to £11.99**	

The main cost savings that are associated with the supply of ethanol-based sampling kits are those associated with the couriering of multiple sampling kits. The shipment of the sampling kit and the return in compliance with ADR regulations can cost as much as £58.87 for a single kit. These costs are markedly reduced (per kit basis) if up to 25 (the maximum ethanol legally shipped per consignment) kits/samples are shipped in each consignment. It is likely that these full costs are not currently being passed onto ecologists looking at small numbers of samples. The use of alternate components in the sampling kits (the use of non-sterile items, the use of non-molecular biology grade ethanol) could save around £4.50 per sampling kit or around 30-35% on the current cost of the components associated with the sampling kit.

The Sterivex filter is a popular enclosed filter system for sampling eDNA and is used as our first example compared to the price of an ethanol precipitation kit (Table 3).

Table 3. Comparison (current) ethanol-based sampling kit with 'sterivex' filter-based kit (price per kit). Courier costs are shown as a range of prices dependent on how many kits are placed into the box (number in brackets), couriering becomes cheaper the more kits placed into the different sized packing boxes/Royal Mail parcels. Total price is again shown as a range due to the different numbers of kits that can be placed into the different sized packing boxes. *This is the total price of the packing materials required i.e. bubble wrap, packing box, kit box etc.

Current ethanol-based sampling kit				Sterivex filter-based sampling kit			
Product	**Supplier**	**Code**	**Price (each)**	**Product**	**Supplier**	**Code**	**Price (each)**
Wrapped gloves	VWR	112-4555	£1.48	Wrapped gloves	VWR	112-4555	£1.48
Biodegradable bag	Polybags	1015150BIO	£0.08	Biodegradable bag	Polybags	1015150BIO	£0.08
Whirlpack bag	Fisher	129-9887	£0.19	Whirlpack bag	Fisher	129-9887	£0.19
Pipette	Fisher	612-4515	£0.56	Sample dipper	Fisher	15488794	£1.72
Sample dipper	Fisher	15488794	£1.72	Sterivex filter	Merck	SVGPL10RC	£10.26
MB Ethanol	Fisher	437435L	£5.02	50mL syringe	Fisher	10636531	£2.18
Sodium Acetate	Merck	S7899	£0.15	10mL syringe	Fisher	15544835	£0.55
50mL tubes	Fisher	10314131	£2.40	N/A	N/A	N/A	N/A
Packaging	Various	Various	£1.91*	Packaging	Various	Various	£1.91*
Binbags for packing boxes	Polybags	Various	£0.23	N/A	N/A	N/A	N/A
Ethanol disposal	Veolia	N/A	£0.56	N/A	N/A	N/A	N/A
Courier Small	DHL	Small (1-3)	£44.58 to £11.50	Royal mail tracker 24 (small parcel)	Royal Mail	1-10 kits	£8.90 to £0.89
Courier Med	DHL	Med (4-9)	£11.50 to £4.95	Royal mail tracker 24 (small parcel)	Royal Mail	11-25 kits	£13.90 to £0.56
Courier Large	DHL	Large (10-25)	£5.88 to £2.35	N/A	N/A	N/A	N/A

Current ethanol-based sampling kit		Sterivex filter-based sampling kit	
Total cost per kit	**£58.87 to £16.64**	**Total cost per kit**	**£32.27 to £18.93**

The sterivex units are costly at £10.26 per unit and RSK ADAS have had supply issues when using on other eDNA based projects. It is noteworthy that the procurement of this type of filter popular in the eDNA monitoring industry, is likely to be slightly more expensive per kit (based on the shipment of 25 kits at a time).

Other methods for eDNA sampling have been described, RSK ADAS report in Task 4 a small study using 33mm PES filters for the recovery of GCN eDNA. A price comparison of these with the sterivex filter unit is provided in Table 4. The alternate filters (which require full validation) could reduce the cost associated with the components within a sampling kit to around £10.00, a 45% saving over ethanol-based sampling kits (based on supply of 25).

Table 4: Comparison of a 'Sterivex' filter-based sampling kit with an alternative PES based filter. Courier costs are shown as a range of prices dependent on how many kits are placed into the box, couriering becomes cheaper the more kits placed into the different sized Royal Mail parcels. Total price is again shown as a range due to the different numbers of kits that can be placed into the different sized packing boxes. *This is the total price of the packing materials required i.e. bubble wrap, packing box, kit box etc.

Sterivex filter based sampling kit				0.45 PES filter-based sampling kit			
Product	**Supplier**	**Code**	**Price (each)**	**Product**	**Supplier**	**Code**	**Price (each)**
Wrapped gloves	VWR	112-4555	£1.48	Wrapped gloves	VWR	112-4555	£1.48
Biodegradable bag	Polybags	1015150BIO	£0.08	Biodegradable bag	Polybags	1015150BIO	£0.08
Whirlpack bag	Fisher	129-9887	£0.19	Whirlpack bag	Fisher	129-9887	£0.19
Sample dipper	Fisher	15488794	£1.72	Sample dipper	Fisher	15488794	£1.72
Sterivex filter	Merck	SVGPL10RC	£10.26	PES Filter (0.45um)	Merck	514-1258	£1.34
50mL syringe	Fisher	10636531	£2.18	50mL syringe	Fisher	10636531	£2.18
10mL syringe	Fisher	15544835	£0.55	10mL syringe	Fisher	15544835	£0.55
Packaging	Various	Various	£1.91*	Packaging	Various	Various	£1.91*
Royal mail tracker 24 (small parcel)	Royal Mail	1- 10 kits	£8.90 to £0.89	Royal mail tracker 24 (small parcel)	Royal Mail	1- 10 kits	£8.90 to £0.89

Sterivex filter based sampling kit				0.45 PES filter-based sampling kit			
Royal mail tracker 24 (small parcel)	Royal Mail	11-25 kits	£13.90 to £0.56	Royal mail tracker 24 (small parcel)	Royal Mail	11-25 kits	£13.90 to £0.56
Total Cost		**£32.27 to £18.93**		**Total Cost**		**£23.35 to £10.01**	

Laboratory cost-benefits

This section does not deal with labour or overheads and only covers cost-benefits to do with the purchase of laboratory consumables. In our experience a change to filtration would not reduce the amount of labour involved in the extraction of DNA from these samples.

Two eDNA extraction methods were highlighted the current DNeasy blood and tissue kit and the DNeasy Power water kit. The prices per sample extraction are £3.54 and £7.97 respectively. It is unlikely that service providers would want to use this alternate kit as routine (higher cost and additional processing steps), but this may be useful with samples that are graded as poor quality (high sediment) as the kit was developed to provide DNA of high quality and yield, even from water sources with high levels of contaminants.

The qPCR mastermixes that are used for eDNA work are the Taqman environmental mastermix and the Taqman fast advanced. For GCN identification using 12 replicates for the GCN analyte and 2 each for the inhibition and degradation control these would cost £33.03 and £28.48 respectively for the 16 PCR reactions that would be required. Further savings can be made when purchasing larger pack sizes for example Taqman environmental mastermix become comparable in price with Taqman fast advanced when the larger pack size is purchased (£26.78 versus £27.12 respectively for 4 x 5 mL). The TMM mastermix identified in Task 3 as having good resistance to three types of inhibitors (Uchii et al. 2019) would cost £18.02 for the 16 PCR reactions that would be required. If any cheaper alternative Taq shows equivalence in terms of assay sensitivity of detecting GCN in environmental samples then a cost saving per sample could be achieved.

If an extra inhibitor removal step was included, for example OneStep PCR inhibitor removal kit (Zymo Research) at a cost of £2.46 per DNA extract to be processed, there could be significant cost benefits to the client. These would arise where any indeterminate samples were put through an inhibitor removal step and re-analysed or where potentially indeterminate samples were put through an inhibitor removal step prior to PCR assay thus avoiding potential re-sampling or traditional surveying costs for the client.

Surveyor/service user cost-benefits

For users of the eDNA kits there could be small price reductions in the cost of a sampling kit if there is a change to ethanol that is not molecular biology grade. However, given that

the market rate for kit assembly and provision is probably no longer a true representation of the real costs it is unlikely that any savings would be passed onto the users.

Significant savings could be made with a move to filter kits as the associated couriering costs are minimal due when large numbers of kits a being sent/returned due to the removal of the need to send via specialist couriers when ethanol kits are being sent/returned. Also, the kits are quicker and easier to assemble so associated staffing costs will be lower meaning that savings could be passed onto kit users.

Where there is a likelihood of high calcium levels in the waterbodies to be sampled significant savings could be made by pre-testing the water with a dip-stick test and/or using a filter-based kit. Previously these would be likely to return an indeterminate result which would mean that the surveyor would potentially have to return to the water body to re-sample or carry out traditional sampling which can be far more costly.

Appendix 1. Ethanol precipitation and filtration comparison (RSK ADAS)

Materials

1) GVS filter technologies syringe filter. PES, 0.45um, 25mm. Product code: FJ25BNPP004AH01
2) GVS filter technologies ABLUO syringe filters, Glass fibre, 33mm, 3.10um.
3) Sterivex PES, 0.22um filter unit (Sterivex SVGP01050)
4) Standard eDNA kits: ethanol and sodium acetate.
5) Luer lock syringes; 50mL and 10mL
6) Caps to seal the filter units.
7) 100% Ethanol
8) Sampling packs (complete with collection bag, gloves, pipette and sampler pot)

Sample Collection

Water was collected from three mesocosms as per the methods used in the technical advice note and either added to ethanol tubes (as per the technical advice note) or 90 mLs filtered through the following filter/s using sterile 50 mL luer-lock syringes and resulting in the samples in the table below.

1. Sterivex 0.22µm filter
2. Sterivex 0.22µm filter in tandem with a glass fibre (GF) pre-filter
3. PES 0.45µm filter
4. PES 0.45µm filter in tandem with glass fibre (GF) pre-filter

Any excess water was pushed through the filters (including the pre-filters which were also kept for analysis) using air in the syringe, then 2 mLs 100% molecular biology grade ethanol added for preservation of the DNA. All filters were sealed with appropriate caps and kept on ice during transport back to the laboratory where they were stored in a fridge at 4°C.

Table A1. Samples generated by ethanol precipitation or filtration of 90 mLs water.

eDNA collection method/ Mesocosm no.	Sample ID
Mesocosm 1. Sterivex 0.22µm filter	M1-1
Mesocosm 1. Sterivex 0.22µm filter + GF pre-filter	M1-2*
Mesocosm 1. Extraction from GF pre-filter used for M1-2 sample collection	M1-2P
Mesocosm 1. PES 0.45µm filter	M1-3

eDNA collection method/ Mesocosm no.	Sample ID
Mesocosm 1. PES 0.45µm filter + GF pre-filter	M1-4*
Mesocosm 1. Extraction from GF pre-filter used for M1-4 sample collection	M1-4P
Ethanol precipitation kit Mesocosm 1	5145
Mesocosm 3. Sterivex 0.22µm filter	M3-1
Mesocosm 3. Sterivex 0.22µm filter + GF pre-filter	M3-2*
Mesocosm 3. Extraction from GF pre-filter used for M3-2 sample collection	M3-2P
Mesocosm 3. PES 0.45µm filter	M3-3
Mesocosm 3. PES 0.45µm filter + GF pre-filter	M3-4*
Mesocosm 3. Extraction from GF pre-filter used for M3-4 sample collection	M3-4P
Ethanol precipitation kit Mesocosm 3	5140
Mesocosm 5. Sterivex 0.22µm filter	M5-1
Mesocosm 5. Sterivex 0.22µm filter + GF pre-filter	M5-2*
Mesocosm 5. Extraction from GF pre-filter used for M5-2 sample collection	M5-2P
Mesocosm 5. PES 0.45µm filter	M5-3
Mesocosm 5. PES 0.45µm filter + GF pre-filter	M5-4*
Mesocosm 5. Extraction from GF pre-filter used for M5-4 sample collection	M5-4P
Ethanol precipitation kit Mesocosm 5	5286

*Note that only the sterivex/PES filters were DNA extracted to create these samples, GF filters were extracted separately.

Laboratory Processing

Ethanol precipitation kits were processed as per the methods in the technical advice note. Filter units were processed as follows:

1. Remove the ethanol from the filters to freshly labelled Eppendorfs and store in fridge at 4°C.
2. Pipette ATL/ proteinase K (DNeasy blood and tissue kit) as follows ensuring good coverage of the filter surface with the lysis buffer.
    - PES 0.45µm filters – 540µL ATL and 40µL proteinase K (pre-mixed)
    - Sterivex 0.22µm filters – 720µL ATL and 60µL proteinase K (pre-mixed)
    - Glass fibre filters – 540µL ATL and 40µL proteinase K (pre-mixed)

3. Seal the filters with suitable caps. Vortex briefly and then secure to a rotator. Leave to incubate over night at 37°C with rotation (slow).

Next day:

4. Centrifuge the ethanol removed from the filter units at 14,000 x G for 30 mins at 6°C then discard the ethanol supernatant and allow to air dry for 10 minutes.
5. Remove the filters from the 37°C incubator and vortex. Recover as much of the ATL/proteinase K mix from the filter as possible into the tube containing the pelleted DNA from the ethanol preservative by pushing out of the filter using a syringe.
6. Add 4-600µL of ethanol to each sample (400µL for PES and glass fibre filters and 600µL for sterivex filters).
7. Continue with the remaining steps of the DNeasy blood and tissue kit as per the manufacturer's instructions.

qPCR assay

All samples were tested for inhibition and then GCN using the methods in the technical advice note. None of the samples were found to show any level of inhibition. GCN results as PCR score (out of 12) and Ct values are shown in Table A2.

Table A2. GCN PCR assay scores and average Ct values.

Sample ID	GCN score (out of 12 reps)	Mean Ct
M1-1	12	34.62
M1-2	6	38.71
M1-2P	10	39.78
M1-3	12	34.16
M1-4	3	38.81
M1-4P	12	33.78
5145	11	37.01
M3-1	12	34.25
M3-2	11	38.75
M3-2P	12	36.0
M3-3	12	34.45
M3-4	10	39.71
M3-4P	12	35.3
5140	12	38.23
M5-1	12	32.88
M5-2	12	36.62

Sample ID	GCN score (out of 12 reps)	Mean Ct
M5-2P	12	32.39
M5-3	12	32.97
M5-4	12	36.35
M5-4P	12	32.61
5286	12	34.22

Appendix 2. Ethanol precipitation versus filtration eDNA scores (NatureMetrics with Atkins and HS2 Ltd)

Table A3. eDNA score, defined as the number of positive qPCR replicates out of 12 qPCR replicates performed on a sample, produced by ethanol precipitation or filtration for each pond in each month that sampling occurred during the project thus far. NS indicates that no sample was collected and analysed in a given month due to ponds being dry or unsafe. Note that for these reasons, sample size decreased from 21 ponds in April to 18 ponds in July, and that some ponds were added in July to bolster sample size for the comparison of eDNA capture methods. These additional ponds cannot be used for investigating out-of-season GCN eDNA detection as there is no in-season data for comparison. EtOH ppt^{n} indicates ethanol precipitation.

Pond ID	Previous GCN status	April		May		June		July	
		EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration
1	N	0	0	0	0	-	-	-	-
2	N	0	0	0	0	0	0	0	0
3	N	0	0	0	0	0	1	0	0
4	Y	1	12	0	12	1	0	0	12
5	Y	1	11	0	12	0	12	0	12
6	Y	0	12	5	12	0	12	1	12
7	Y	0	1	3	7	0	0	7	12
8	Y	0	7	10	1	4	12	12	12
9	Y	9	10	3	12	1	12	4	0
10	Y	2	12	12	12	12	1	12	11
11	Y	6	12	10	2	12	12	12	12
12	Y	12	12	12	7	6	12	11	11
13	Y	2	8	12	12	12	2	11	0
14	Y	0	6	5	12	0	11	4	12
15	Y	12	0	12	12	12	11	12	0
16	Y	0	12	0	1	NS	NS	NS	NS
17	Y	2	9	NS	NS	NS	NS	NS	NS
18	Y	1	12	1	7	0	12	1	12
19	Y	3	12	0	12	12	12	NS	NS

Pond ID	Previous GCN status	April		May		June		July	
		EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration	EtOH ppt^{n}	Filtration
20	Y	1	12	8	12	11	12	12	2
21	Y	0	9	10	12	NS	NS	NS	NS
22	N	NS	NS	NS	NS	NS	NS	0	0
23	Y	NS	NS	NS	NS	NS	NS	12	12

Appendix 3. Summary of GCN sampling protocol review questionnaire

1. Are you directly involved in collecting water samples for eDNA analysis of GCN presence/absence?

There were 76 yes responses and 1 no response.

2. Please rate the following statements.

Statement or question asked	Strongly Agree	Agree	Neither Agree or Disagree	Disagree	Strongly Disagree
I find the protocol easy to follow	64.9%	33.8%	1.3%	N/A	N/A
There is enough emphasis on collecting as clean a sample as possible	41.6%	41.6%	7.8%	9.1%	N/A
Would you pay extra for 100% recyclable consumables?	65.3%	28%	5.3%	1.3%	N/A
Would you be happy to use a smaller transfer pipette to save on plastics?	63.6%	22.1%	3.9%	7.8%	2.6%
Would you be happy to use the bag containing the ladle to collect the water in rather than a separate bag?	74.4%	19.2%	2.6%	2.6%	1.3%
Would you be happy to use the ladle rather than a transfer pipette to move the water into the tubes of preservative to save on plastic?	39.7%	19.2%	3.8%	29.5%	7.7%
Would you be prepared to let the sediment in your water sample settle in the bag for 10-20 minutes to improve sample quality?	20.5%	35.9%	10.3%	23.1%	10.3%
Do you find it easy to sample wearing gloves?	32.5%	36.4%	15.6%	11.7%	3.9%

Question asked	Results
3. Did you know that the current shelf life of the kits is 3 months?	Yes: 36 No: 42
4. Do you routinely stir the water column prior to sampling as stated in the technical advice note?	Yes: 70 No: 8
5. If yes to the above, does this noticeably decrease the quality of the sample?	Yes: 31 No: 39
6. For samples containing a large amount of sediment would you be willing to pour the water through a 'coffee type' filter or fine mesh?	Yes: 72 No: 5
7. Are you aware of how sediment and/or algae can affect our ability to generate results?	Yes: 47 No: 30
8. Are you aware that certain types of water chemistry (likely high calcium content) can react with the preservative and interfere with our ability to generate results?	Yes: 21 No: 57
9. Did you know that in this situation a filter kit could be used to sample the water?	Yes: 9 No: 69
10. Have you ever used a filter to collect water samples?	Yes: 6 No: 72

11. If yes to the above, how does this compare to the ethanol preservative kits in ease of use?

ID	Responses
7	Find filters more fiddly but willing to use if it increases reliability
9	Its a bit more time consuming but does allow for a better sample
12	Comparable. The 6 x replicate tubes of ethanol is a pain though!
13	more fiddly to start with but after getting use to it- its fairly straightforward
14	If they are those that were used in early days of eDNA with a syringe, they were very hard to use due to the pressure needed to push the sample through the filter. Quite often you would have to push the syringe against the floor or a tree.
15	The instructions were more difficult to follow and process was more fiddly. I think accompanying diagrams to the instructions and possibly an instruction video on RSK ADAS website would be useful.

12. What do you do with unused kits?

ID	Responses
1	Re use on other projects or training where possible.
2	Return to lab or use on other projects
3	Return to provider
4	Return along with used kits
5	Return to labs at end of eDNA season
6	Attempt to send back to the lab
7	Use for another survey if before the expiry date or return to eDNA.
8	They just get left piling up in the office!
9	Keep them for other projects or try to send them back.
10	Dispose of them in a waste bin but not in a common household/office bin as the water sample bag may contain bacteria
11	Sent them back (this year), previous years have kept them.
12	save for training/keep collection equipment (not alcohol vials) as spares, then eventually throw them away
13	Send back asap or re-distribute to other projects
14	return or use on other jobs
15	Use them for training
16	Keep them for further use or return to you
17	Send them back - we try to reuse the plastic aspects where possible (for non water based reasons)
18	Save for another job or send back if outside of GCN season.

ID	Responses
19	These will be sent back to cellmark.
20	return to supplier
21	Send back to lab unused if not needed for another job. Keep and use on a different job if possible
22	donate to local ARG group or bin.
23	Use for training purposes or return to adas
24	Keep for later date.
25	We don't usually have any
26	disposed of in general waste after 3 months
27	Use on another job if possible
28	Save for another project
29	Send them back
30	Use them on another site
31	Return them to store depot
32	Return to client
33	We either send back to ADAS or let the ethanol dissolve and hold onto the sampling kits for use in an emergency the following year (the lab once ran out of sampling kits so we used a spare from the previous year as was all still sealed and therefore sterile)
34	Post them back to supplier.
35	Either send them back to supplier or keep some back for training purposes
36	Usually end up on another job we have or sent back to ADAS (rare)
37	Return them to the office to be used on other jobs
38	I have stored an unused kit in a dark place
39	Return to ADAS
40	company can send back but try to use on other projects
41	Returned them to Natural England
42	I thought they all had to be used by the end of June as per the guidance, but some unused ones in the past have been sent onto me and I've done extra ponds
43	Return them to the office to be used on another job
44	Can we return them for a refund?
45	At the moment we have them in storage, however we have emailed NE to ask if we can send them back
46	N/A
47	Give them back to the organisation who asked me to collect the sample

ID	Responses
48	Return to the lab
49	try to reuse within company
50	Use on other sites or empty and use as sample pots
51	Send them back to the lab
52	Tbc! Probably returning them
53	Keep them in the office for too long. Have a tidy up and throw them away (recycling where possible)
54	In 2021, an unused kit was used on another pond. In 2022 all kits were requested to be returned.
55	bin them
56	Return to lab
57	Re use
58	send them back to provider
59	Return them to ADAS
60	N/A
61	dont have any unused kits
62	Send them back or bin them
63	Returned them to the lab
64	If in date offer to other members of RSK Biocensus or send back to ADAS.
65	Send them back to the lab.
66	all kits have been used so far
67	Try to reuse on other sites where possible. Kits left at the end of the season tend to be thrown away as we are aware that the shelf life will not extend to the following season.
68	Return to supplier
69	If unopened/ unspoiled we post back to Cell Mark
70	Natural England requested return to laboratory
71	Dispose of them - try and recycle where possible
72	Return to ADAS
73	Bin
74	keep in store room
75	"Return to Sender" (Elvis 62)

13. How much of the sampling kit do you recycle?

ID	Responses
1	None to very little.
2	Very little - would like to do more
3	All plastics
4	I put it all back in the box as packing prior return to the lab
5	None currently, as I wasn't aware it could be recycled safely
6	None, although it usually all gets sent back when I've done it before. Anything left, if recyclable, would be recycled
7	Everything that can be recycled
8	soft plastics (through a trial recycling scheme at our local Tesco), blue sample collecting pot, paper from glove packets
9	As much as possible, however its rare to find the type of plastic recycling when on Site
10	Mainly the paper for the gloves
11	Dependent on location. If returning home / to the office as much as possible. If staying away, less as its not as easy in a hotel.
12	none
13	All the recyclable items.
14	not enough :(
15	None
16	None
17	The main bag - unsure whether we can recycle it all
18	I can't speak for my other team members but I recycle the bags with supermarket plastic bags, pipettes with kerb-side and try to save and reuse the ladles for other things in my own home
19	some but not always possible
20	As much as possible. All plastics are separated accordingly and the cardboard is composted. The postage bags are relabelled for other postage.
21	Everything that is recyclable. However, that is not to say that it all goes on to be recycled though.
22	Only the outer bag which says it's recyclable
23	just the cardboard
24	20%
25	As much as possible
26	None apart from the boxes
27	cardboard packaging is recycled. sampling kits are retained and used as training aids

ID	Responses
28	None
29	not much of it because its all soft plastic which cant be recycled where we are based
30	Just the boxes and paper instructions
31	As much as we can. Also re-use ladle pots as they are useful
32	As much as possible
33	Soft plastic and lid.
34	All of the hard plastic and cardboard but the thinner plastic bags tend to go in the bin. Zip-log bags are saved to collect aquatic plant material for ID.
35	The cardboard, paper, and hard plastic elements (e.g. pipette, ladle)
36	All
37	as much as possible
38	as much as possible, which as far as I'm aware is just the paper package that the gloves come in
39	Most of the kit. Soft plastics (bags- at Tesco's) the other bits, ladles, pipettes will be sterilised to be used again. Rubber gloves can be used again, although will not be recycled.
40	Unsure
41	none
42	All of the plastic bags reusable for transporting other samples, fish etc, ladles and pipettes were taken by an employee for his children and home schooling
43	Everything apart from the plastic ladle, gloves and pipette. The soft plastics can go in the recycling at supermarkets and the cardboard box in household recycling.
44	Anything that can be recycled
45	none
46	The soft plastic, but we have to take it to a recycling point in a supermarket and even them I am not sure if it is fully recyclable as some has pond water on it. the amount of plastic waste was the biggest concern of mine throughout the sampling.
47	None
48	Everything that can be recycled
49	None of it.
50	We saved the tubes and pipettes for the local home-schooling network
51	none
52	try to do 90%, but not sure if it actually is recyclable
53	Cardboard and plastic when possible
54	As far as I know little is recyclable locally, but I compost the paper and cardboard components
55	As much as possible

ID	Responses
56	100% if possible
57	none
58	As much as possible
59	All I can
60	none
61	Not much
62	The cardboard boxes and paper instructions
63	The boxes, the bags, the pipette
64	Most of it, but not clear if it looks like medical waste.
65	Recycled some if the plastic bags when I found out we could recycle them with plastic shopping bags
66	All recyclable items
67	Just the outer cardboard box
68	paper and cardboard packaging
69	All if able to use on other sites, otherwise only cardboard / paper as unsure of which of the plastics are possible to recycle
70	Only outer bag which can be recycled, everything else has to be thrown away
71	I recycle the soft plastics and the cardboard and paper. I throw away the Perspex type ladle and lid and the rubber gloves. The ladle lid is entirely unnecessary..
72	As much as possible
73	Cardboard but not tubes as these aren't recyclable are they?
74	as much as possible -however the plastic packaging at the individual parts come in is not recycled in our area
75	All when possible
76	as much as possible although not sure if the office bins get correctly recycled
77	Paper and cardboard

14. Do you think there should be flexibility in the start and end of the season?

There were 17 yes answers, 10 no answers, and 51 maybe answers.

15. If so, what factors should feed into this e.g. local knowledge, GCN activity in the area?

ID	Responses
1	Weather conditions. Newts can enter ponds much earlier and stay much longer than established guidance
2	GCN activity - known that they enter ponds a lot earlier than the season starts. Site information and previous survey results may also help
3	Weather

ID	Responses
4	Local knowledge and records of GCN in the area, and their activity - i.e. if they are known to be active earlier in the year in warmer parts of the country, the season could start slightly earlier etc.
5	Experience in my industry is that if you allow more (e.g. starting before mid-April as everyone wants to start pop. estimate surveys if needed asap), consultancies will take more. But if any variation is scientifically robust and defensible, and the guidance is clear, then yes!
6	confirmed GCN presence e.g. sighting of GCN in pond, eggs found
7	Geographic location and weather - southern sites could probably start earlier
8	Local knowledge
9	Local knowledge, temperature consistency
10	Not sure
11	I do not know enough about GCN ecology to know
12	Whether previous eDNA has confirmed presence prior to this season - GCN in the area
13	Local and landowner knowledge, records of GCN in local area, timing of instruction by clients
14	Late surveys will only favour GCN breeding ponds (where larvae are present and shedding DNA) - therefore the window should not be extended. GCN in Cheshire seem to return to ponds in March, so 15th April could be brought forward to 1st April
15	local knowledge of GCN activity in the area.
16	GCN activity. GCN seen in ponds as early as March this year.
17	History of the site regarding GCN etc, recent weather fluctuations (excessive heat likely to dry ponds etc)
18	The earliest you can get out to some of the more overgrown ponds the better as vegetation has not peaked
19	The weather and timing of the season each year
20	Weather conditions, local knowledge, GCN activity
21	The abundance of GCN in the area and possibly spring temperatures for an early start to the season.
22	Evidence of early season breeding (March). GCN occupancy of a pond beyond Late June
23	I think a couple of days leeway is fine but validity for panning applications is not as good. Negative results can't be trusted after the season has ended but positives are still respected.
24	GCN activity, maybe weather conditions
25	records in the area
26	I've found GCN eggs in early March and mid-July. So yes, it would be much easier to stretch the time frame.
27	Which zone you are in/ Where abouts in the country you are. Climate.
28	GCN activity
29	No idea.

ID	Responses
30	there is a simplicity in having fixed dates, and this is easy to explain to clients (wriggle room on dates may not be helpful from a client perspective)
31	Local knowledge of the GCN activity
32	I would probably start earlier in the year but I'd prefer to defer to your experience of how GCN presence/eDNA detectibility varies. If I'm torching I need it to be dark and I'm mostly looking for adults, eDNA can be done in daytime and will detect efts and eggs
33	local weather conditions and populations. i.e. places like Kent and Devon and Cornwall see breeding a lot earlier
34	Weather, temperature,
35	Local weather and overnight temperatures
36	It takes us all sampling time to do the ponds, we could potentially start earlier in East Kent as season starts early
37	Science. If there are larvae in the pond will there be eDNA
38	Local knowledge
39	Seasonal weather - GCN seem to be moving into ponds earlier therefore there should be a week or so flexibility with start date rather than 15th April which often falls on a awkward working week.
40	Highly dependent on location in UK and weather. Personally, I prefer strict dates for consistency.
41	weather
42	I am aware that some areas see GCN in ponds from before the season begins and afterwards, but I guess this is largely weather dependent and sampling protocols must be based on the most robust chances of confirming results
43	Both of the above and recent local weather conditions, pond aspect/ type/ proximity to hibernacula etc...
44	Local knowledge / weather
45	Weather, professionally judgement
46	GCN activity

16. How do you think the collection kit/procedure could be improved?

ID	Responses
1	Less packaging
2	Its a very straightforward process, all info on how to return the kit is clear, and provision of necessary labels helps
3	I generally think it is fine as it is. I think the option to remove sediment should be an optional extra with the kits.
4	I think it's OK as it is.
5	Less plastic, and clear instructions on what can and can't be recycled from the kits
6	I think it's pretty good! Can't think of anything off the top of my head
7	Less plastic (could corn starch be used instead of plastic)

ID	Responses
8	Can be hard to tip the sample from the collection pot into the bag using the long handle. I'm not sure there needs to be a glove change between collection of samples and pipetting it into the preservatives. There's way too much waste (mainly packaging) at the moment.
9	I was happy with what was provided, maybe less plastic
10	Less plastic
11	less waste
12	all good except the plastic waste :)
13	recycling - less plastic - recyclable piece of paper with instructions within the parcels
14	Much less single-use plastic or kit easier to recycle. Swirling of water column often difficult in shallow/high sediment water so good samples difficult to attain. Lids on ethanol samples often leaky.
15	Addressed above - alter the protocols to help reduce waste. Include a way of sealing the sample for settlement at home/office, but not in the field (lots of ponds, not too much time to spare).
16	Is is necessary to provide samples into 6 vials? Could one vile be provided out in the field and returned, then the lab provides 6 sub samples? Vials could be re-used - autoclave plastic or glass vials. Need to accept that the protocol is incorrect in assuming that 20 samples can be taken without entering the pond - often a fringe of vegetation means that you have to submerge your feet to reach open water.
17	Use of a calendar to book delivery adn collection slots?
18	Less packaging.
19	If there was a way the samples could be collected easily rather than posted, that would be very useful!
20	As above, less plastic/biodegradable/reusable kit where possible.
21	Some method of minimising the admin for each kit: labelling each individual tube, and filling in the form for each set of samples
22	Instructions on box, recyclable contents.
23	Making more of the kit able to be recycled and to provide a filter to help with waterbodies where high amounts of sediment are present.
24	Less writing on test tubes - time consuming!
25	I don’t think it needs to be
26	no
27	More recyclable materials, less wastage
28	Perhaps information on who could deliver the kits to the lab, for new testers. I spent time looking for a courier service to pick up my kits. Most wouldn't carry them due to the contents, I ended up using Royal Mail, which was cheaper and ok with the contents. I was quoted £250 by one courier service.
29	n/a works well
30	Less waste
31	I had to buy an extendable flagpole (like those used at festivals) to utilise a fishing rod and then tape the ladle on the end to get water samples. It's not possible to reach the water on some restoration ponds due to steep sides or banks of bramble.

ID	Responses
32	Plastic wastage lower (you don’t need a cap for the ladle, wasted plastic)
33	There are always 2 people on the survey so 2 pairs of gloves could be provided. It's not always possible to stir the water column if the water level is very low so a filter would be good. The procedure should talk about best practice if an extension cane is used
34	-
35	I don't have anything to add here. It would be nice if there wasn't so much plastic.
36	mostly excellent, occasonally awful!
37	all recyclable + info regarding samples with sediment how to reduce but still get a result.
38	Reduce plastic waste where possible.
39	I have fairly severe hand and wrist arthritis. I find the multiple labelling of the tubes etc v difficult and this should not be needed as there is a barcode system. I also find the pipetting v painful so would prefer a larger pipette or to pour and/or fewer replicate tubes. The recycling issue is a red herring, while I favour minimising waste, recycling and reuse, environmentally it is irrelevant in this case, cf the amount of fuel likely consumed gathering the samples!
40	Less equipment per kit as sometimes we have to carry multiple kits over a long distance to reach the ponds. Less plastic would be good. We have also started using a long-handled pole which we attach the ladle to as sometimes we can't reach the water’s edge otherwise. Our main issue with collecting water samples has been pond access not the eDNA kits. Overall, we find this process straight forward and practical.
41	yes
42	Perhaps a choice of glove size. The ones in the kit were much too big for me and hindered me when using the pipette.
43	It’s pretty straight forward - I wrap them and take them to the post office
44	Advice on sampling using an extender to access deep water, etc
45	It’s has been the first year for me sampling and I found it OK, struggled slightly to get samples on some ponds which were shallow on the edges
46	Possibly offer drop off points for labs/offices close to the M1 motorway (midlands).
47	Make more of kit recyclable, have less 'pieces' (using ladle instead of syringe and ladle bag are great ideas), a longer ladle/extension piece for safety when collecting samples, tighter fitting gloves.
48	If labels could be pre-printed so we don't have to write on the kits in the field and can't forget to include the right info. Often two people are carrying out the sampling together and only one pair of gloves, make clear in instructions which parts can/can't be touched without gloves.
49	Service is great but maybe some stronger gloves would be better as I frequently end up tearing them when moving round ponds that have very scrubby areas!
50	Less individually packaged equipment, no lid for the sampling ladle as it's rarely used. A filter for some water samples could be good, especially for turbid ponds/ponds with a lot of duckweed etc
51	We don't need the lid for the ladle. The ladle could be recyclable plastic. The bag the gloves or ladle come in could be used to collect the water and/or to contain the sample box when posting back. We don't need the two pieces of A4 paper in each kit, but the blank side of one of the pieces of paper could be used for instructions on what/ how to post back and

ID	Responses
	what to recycle. We don't need the spare ethanol sticker in each kit. If posting back a box full of three sample kits, I cut out the Cell Mark address and ethanol sticker from the postage envelop and stick it to the box for posting.
52	Too much plastic. Pipette doesn't hold enough volume. Having a sample kit that could safely stand up on uneven ground and not tip to allow individual sampling would be really helpful. Regarding question 3 Natural England have previously stated kits are viable for longer than 3 months
53	I think it works well at the moment
54	DHL don't always communicate well, area usually late and some drivers cannot find our office
55	Postage should be covered for returning unused kits.

Appendix 4. Water chemistry analysis

Table A4. Elevated levels of Calcium are highlighted in bold text.

Lab Sample ID	17/05396/1	17/05396/2	17/05396/3	17/05396/4	17/05396/5	17/05396/6	17/05396/7
Client Sample ID	Pond Z Water	P4 Water	P6 Water	P3 Water	D2 Water	Meso-cosm 2	Arbore-tum pond
Date Sampled	17-Jul-17	17-Jul-17	17-Jul-17	17-Jul-17	17-Jul-17	01-Aug-17	03-Aug-17
Sample Type	Water - EW	Water - EW	Water - EW	Water - EW	Water - EW	Water - EW	Water - EW
DOC (w) mg/L	7.4	1.9	2.3	1.1	7.5	8.5	16.7
DIC (w) mg/L	5.9	1.8	0.3	<0.2	56.3	18.8	45.7
Calcium* mg/L	**216**	**311**	**399**	**352**	**410**	20	69
Magnesium* mg/L	36	29	35	32	25	6	21
Potassium* mg/L	25	16	16	20	22	8	14
Sodium* mg/L	64	26	26	26	34	12	12

*dissolved

Appendix 5. Comparison of 14,000xg and 5,000xg centrifugation

Table A5. PCR score and average Ct achieved with different centrifugation speeds.

Sample	14,000xg GCN result	14,000xg GCN average Ct	5,000xg GCN result	5,000xg GCN average Ct
Wantage 2	7/12	41.9	7/12	39.91
Old Sodbury	3/12	39.91	2/12	39.75
Wantage 1	12/12	33.13	12/12	33.8
HM1	9/12	39.04	12/12	38.74
HI0	3/12	39.58	6/12	40.65
HM3	12/12	33.8	12/12	33.53
Callow Hill	12/12	38.45	11/12	37.44
FH8	12/12	36.03	9/12	35.54
N5	3/12	39.74	11/12	38.58
H3	10/12	39.76	9/12	39.76
H1	12/12	37.5	12/12	36.54
P2	12/12	33.88	12/12	33.36
P3	8/12	39.16	9/12	39.36
M2A	12/12	36.11	12/12	37.4
M1A	5/12	39.9	4/12	42.2
M2B	12/12	37.17	11/12	39.4
P4	12/12	38.68	10/12	39.34
P1	12/12	38.31	12/12	38.37
M2C	11/12	39.11	12/12	38.8
M1B	6/12	39.8	7/12	39.6
M1C	6/12	41.2	12/12	38.7

Appendix 6 Questionnaire to answer Task 4 - Via consultation create a list of any projects underway looking at GCN protocols and /or methodologies

1. Have your laboratories performed any direct comparisons between ethanol precipitation and filtration methods for GCN detection?
2. If yes to the above, would you be willing to share this information with ADAS directly to eDNA@adas.co.uk or via Natural England so that Natural England can forward to us if you would like to remain anonymous?
3. Do you have any direct comparisons between ethanol precipitation and filtration methods for species other than GCN which could be used to inform this review that you would be will to share with ADAS directly to eDNA@adas.co.uk or via NE so that Natural England can forward to us if you would like to remain anonymous?
4. What do you think the main advantages of ethanol precipitation or filtration have over each other?
5. In your experience are both methods suitable for all pond types?
6. In terms of ethanol sampling kit shelf life, Natural England changed this to 3 months from the original 1 month after eDNA service providers shared data with Natural England in 2017. Do your laboratories have any additional information on kit shelf life generated since 2017? This would also help to reduce wastage.
7. If yes to the above, would you be willing to share this information with ADAS directly to eDNA@adas.co.uk or via Natural England so that Natural England can forward to us if you would like to remain anonymous?
8. Aside from filtration, have you trialled any new technologies (equipment or reagents etc.) since 2014 for the application to GCN detection?
9. Natural England would be interested in understanding any projects that are currently underway that address methods and protocols used in GCN eDNA analysis. Is your laboratory currently engaged in GCN eDNA method development?
10. If yes to the above, would you be willing to share this information with ADAS directly to eDNA@adas.co.uk or via Natural England so that Natural England can forward to us if you would like to remain anonymous?
11. Finally, do your kits make best use of recyclable plastics wherever possible and/or how have you reduced plastic wastage?

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