Evidence review on the use of environmental DNA (eDNA) for detecting the presence of great crested newts in waterbodies

Evidence Report No. 989

Author name: Richard A. Griffiths

Author affiliation: Durrell Institute of Conservation and Ecology, School of Natural Sciences, University of Kent, Canterbury, Kent

About Natural Resources Wales

Natural Resources Wales' purpose is to pursue sustainable management of natural resources. This means looking after air, land, water, wildlife, plants and soil to improve Wales' well-being, and provide a better future for everyone.

Evidence at Natural Resources Wales

Natural Resources Wales is an evidence-based organisation. We seek to ensure that our strategy, decisions, operations and advice to Welsh Government and others are underpinned by sound and quality-assured evidence. We recognise that it is critically important to have a good understanding of our changing environment.

We will realise this vision by:

- Maintaining and developing the technical specialist skills of our staff;
- Securing our data and information;
- Having a well-resourced proactive programme of evidence work;
- Continuing to review and add to our evidence to ensure it is fit for the challenges facing us; and
- Communicating our evidence in an open and transparent way.

This Evidence Report series serves as a record of work carried out or commissioned by Natural Resources Wales. It also helps us to share and promote use of our evidence by others and develop future collaborations. However, the views and recommendations presented in this report are not necessarily those of NRW and should, therefore, not be attributed to NRW.

Report series: NRW Evidence Reports

Evidence report no: 989

Publication date: March 2026

Contract number:	EPPES-001
Contractor:	Emeritus Professor Richard A Griffiths FRSB, Durrell Institute of Conservation and Ecology, School of Natural Sciences, Marlowe Building, University of Kent, Canterbury, CT2 7NR, UK
Contract manager:	E Sharps
Title:	Evidence review on the use of environmental DNA (eDNA) for detecting the presence of great crested newts (GCN) in waterbodies
Author(s):	Professor Richard A Griffiths
Quality assurance:	Internal peer review.
Peer reviewer(s):	S Dyer
Approved by:	J Hanson

Distribution list

Core distribution list managed by the Library: National Library of Wales, British Library, Welsh Government, NatureScot, Natural England

Other organisations and individuals this report has been distributed to by the authors or contract manager:

Recommended citation for this volume:

Griffiths R.A. 2026. Evidence review on the use of environmental DNA (eDNA) for detecting the presence of great crested newts (GCN) in waterbodies. NRW Evidence Reports Report No: 989, 25pp, Natural Resources Wales, Bangor.

Contents

1. Crynodeb gweithredol

Mae DNA amgylcheddol (eDNA) wedi dod i gael ei ddefnyddio'n helaeth i ganfod presenoldeb neu absenoldeb tebygol rhywogaethau mewn systemau dyfrol. Yn achos y fadfall ddŵr gribog (*Triturus cristatus*), mae eDNA yn aml wedi perfformio'n well na dulliau arolygu traddodiadol sy'n gofyn am arsylwi gweledol. Fodd bynnag, fel pob techneg arolygu, mae canfod eDNA yn destun amrywiadau tymhorol a pherfformiad amherffaith, sy'n golygu y gellir cofnodi canlyniadau negatif ffug a phositif ffug.

I gefnogi rôl CNC yn cynghori datblygwyr ac ymgynghorwyr ecolegol, asesodd yr adolygiad hwn y dystiolaeth wyddonol gyfredol ochr yn ochr â 14 o adroddiadau ecolegol a gyflwynwyd i CNC a oedd yn cynnwys canlyniadau eDNA ar gyfer *T. cristatus*. Roedd y nodau fel a ganlyn: (1) gwerthuso technegau maes a labordy sy'n effeithio ar ganfyddadwyedd y rhywogaeth gan ddefnyddio eDNA, ac (2) adolygu patrymau tymhorol o ganfod drwy eDNA i lywio'r arweiniad ar y ffenestr arolygu a argymhellir.

Ar hyn o bryd mae CNC yn ei gwneud yn ofynnol i arolygwyr ddilyn protocol WC1067 (Biggs et al. 2014) pan fyddant yn cynnal arolygon eDNA ar gyfer y fadfall ddŵr gribog. Fodd bynnag, mae datblygiadau technolegol ers sefydlu'r protocol yn golygu y gallai diweddaru'r arweiniad wella dibynadwyedd. Mae dull samplu drwy hidlo, er enghraifft, yn caniatáu i fwy o ddŵr gael ei brosesu, a gallai gynyddu'r tebygolrwydd o ganfod y rhywogaeth. Byddai symud i brotocol newydd yn gofyn buddsoddi mewn hyfforddiant, cyfarpar a gwaith sicrhau ansawdd, a gallai fod yn heriol cymharu canlyniadau ar draws gwahanol ddulliau.

Mae protocol WC1067 yn pennu ffenestr arolygu rhwng 15 Ebrill a 30 Mehefin. Er y gellir canfod eDNA y tu allan i'r cyfnod hwn, nid yw'r crynodiadau'n olrhain yn syml nifer y madfallod llawndwf. Pe bai'r ffenestr arolygu'n cael ei chwtogi i ddiwedd mis Mai, mae perygl o fethu'r cyfnod pan fo twf larfâu yn achosi cynnydd yng nghrynodiadau eDNA. I'r gwrthwyneb, pe bai'r arolygon yn cael eu hymestyn hyd at ddiwedd yr haf neu'r hydref, mae tebygolrwydd cynyddol o ganlyniadau negatif ffug wrth i'r eDNA ddadfeilio neu wasgaru. Ar y sail hon, dylid cadw'r ffenestr arolygu a argymhellir, sef rhwng 15 Ebrill a 30 Mehefin. Y tu allan i'r cyfnod hwn, gall canlyniadau eDNA positif ddal i ddangos presenoldeb, ond ni ddylid trin canlyniadau negyddol fel tystiolaeth o absenoldeb onid oes cefnogaeth i hynny o ddata arolwg a gasglwyd o fewn y ffenestr arolygu a gymeradwywyd.

Nid yw'r dystiolaeth gyfredol yn dangos unrhyw berthynas ddibynadwy rhwng crynodiad eDNA a maint y boblogaeth. Gall niferoedd oedolion fod yn anghyson, a chaiff lefelau eDNA eu dylanwadu gan nifer o newidynnau amgylcheddol, sy'n golygu nad yw'r naill ddull na'r llall yn cynnig amcangyfrif dibynadwy o helaethrwydd y boblogaeth.

At ei gilydd, mae eDNA yn fwy cost-effeithiol ac, ar y cyfan, yn fwy dibynadwy na thechnegau arolygu gweledol traddodiadol ar gyfer pennu presenoldeb neu absenoldeb tebygol. Dim ond gan ddarparwyr gwasanaeth sydd wedi cwblhau profion hyfedredd yn llwyddiannus y dylid derbyn canlyniadau, er y gall fod rhywfaint o amrywio rhwng labordai o hyd.

Mae meta-bargodio, sy'n galluogi canfod rhywogaethau lluosog, yn disodli dull qPCR un rhywogaeth fwyfwy mewn ymchwil ecolegol. Fodd bynnag, gall meta-bargodio fod yn llai sensitif na qPCR pan fo crynodiadau eDNA yn isel iawn, a all gyfyngu ar ei ddefnyddioldeb mewn rhai sefyllfaoedd.

Roedd yr adroddiadau a gyflwynwyd i CNC yn amrywio'n fawr o ran manylder. Er mwyn sicrhau y gellir dehongli canlyniadau eDNA yn gyson, dylai'r holl ddata arolwg a gyflwynir gyda'r protocol WC1067 gynnwys gwybodaeth am asesiadau cyn-samplu, rheolaethau positif a negatif, ataliad neu ddirywiad, risgiau halogiad, a nifer yr atgynhyrchiadau PCR a ddychwelodd ganlyniadau positif.

2. Executive summary

Environmental DNA (eDNA) is an increasingly widely used method to detect the likely presence or absence of a species in a system. It frequently out-performs traditional survey methods that require direct observation, and the great crested newt (*Triturus cristatus*) has been at the heart of research and development in this area. Like all survey methods, however, eDNA may be prone to seasonal fluctuations and imperfect detection, occasionally returning false negatives or false positives in survey results.

Together with 14 ecological reports containing eDNA results submitted to NRW, the current literature on eDNA surveys for *T. cristatus* was reviewed to support NRW's role in providing guidance to developers and consultants undertaking eDNA surveys for the species. The objectives were twofold: (1) to review the scientific evidence of the field and lab techniques relating to the detectability of *T. cristatus* using eDNA; (2) to review the seasonal detectability of *T. cristatus* using eDNA with a view to improving guidance on the appropriate survey window.

NRW currently requires surveyors to adopt the WC1067 protocol (Biggs et al. 2014) when carrying out eDNA surveys for great crested newts. However, the past decade has seen considerable improvements and refinements in eDNA technology and updating this protocol may result in more reliable detection of the species (Rees 2023). In particular, filtration is likely to increase the volume of water from a pond that can be sampled. However, adoption of a new protocol by service providers would require investment in training and equipment. Equally, direct comparisons of eDNA results obtained by different protocols may be difficult.

Although the WC1067 protocol requires eDNA samples to be taken between 15 April and 30 June, eDNA may be detected outside of this survey window. Fluctuations in eDNA concentrations are not simply related to adult abundance. Reducing the survey window to the end of May risks missing a period when eDNA concentrations are increasing due to the growth and development of larvae. Equally, extending the survey window into late summer or autumn increases the risk of false negative results. We therefore recommend that the survey window is retained as 15 April-30 June. Outside of this window, positive eDNA results may be accepted as evidence of presence but negative eDNA results should not be accepted as evidence of absence without additional data obtained during the recommended survey window.

As simple counts of adult *T. cristatus* may give unreliable estimates of population size and eDNA concentrations are also affected by a range of environmental factors, there are currently no consistent relationships between eDNA scores and newt population size.

eDNA surveys are generally more cost-effective and reliable than traditional visual survey methods to establish likely presence or absence. Results of eDNA analyses should only be accepted from service providers that have undergone proficiency testing. That said, there may remain some variability between laboratories in providing accurate eDNA results.

Because of its ability to detect multiple species simultaneously, metabarcoding is largely replacing single-species qPCR as the tool of choice in ecological studies. However, metabarcoding may currently be less sensitive than qPCR in detecting very low concentrations of eDNA of a target species.

Reports of eDNA surveys submitted to NRW varied in the detail of the methods used and the results presented. To interpret eDNA results reliably, all reports that have applied the WC1067 protocol should consistently report pre-sample assessment, positive and negative controls, evidence of inhibition, degradation or contamination, and the number of PCR replicates that were positive.

3. Introduction

Environmental DNA (or eDNA) refers to fragments of DNA that are shed by organisms into the surrounding environment through shed skin, faeces, cells, tissues or body fluids. The great crested newt (*Triturus cristatus*) has been at the forefront of research to formulate and test protocols of eDNA sampling that can provide information on the possible presence of the species in ponds. There are currently two main methods by which this can be achieved: (1) using quantitative Polymerase Chain Reaction (qPCR), with specific PCR primers and hydrolysis probes that will only detect and amplify *T. cristatus* DNA; (2) extracting, amplifying and sequencing all DNA present in a sample and then comparing the reads of sequences to those of known taxa in a bioinformatic pipeline (metabarcoding). As both methods rely on the detection of DNA released and left behind by living organisms, they are indirect measures of the presence (or presumed absence) of the species. Inferences about the presence of the species based on eDNA therefore require an understanding of the production and degradation or removal of DNA in natural systems.

The production and release of DNA may depend on the biomass of the species, metabolic rate, activity and life history. The removal of DNA will depend on a variety of environmental factors, including weather conditions, season, UV, substrate type, microbial activity and water quality and flow. Ultimately, the ability to detect the species will therefore depend on the balance between production and removal of DNA from the system. This balance will vary spatially (i.e. between ponds and landscapes) and temporally (i.e. between seasons).
Although eDNA is an indirect measure of species presence or absence, it is highly sensitive and able to detect species when other traditional visual methods fail. It is therefore increasingly being incorporated into routine monitoring (e.g. Biggs et al., 2014, 2015; Fediajevaite et al., 2021; Sun et al., 2024). In common with traditional methods, eDNA is subject to false negatives and false positives, but if the sampling protocol is carried out rigorously the level of imperfect detection is likely to be much lower than in traditional visual surveys.

This review is intended to support NRW's role in providing guidance to developers and consultants undertaking eDNA surveys for great crested newts (*Triturus cristatus*). The objectives were twofold: (1) to review the scientific evidence of the field and lab techniques relating to the detectability of *T. cristatus* using eDNA; (2) to review the seasonal detectability of *T. cristatus* using eDNA with a view to improving guidance on the appropriate survey window. As the delivery of both objectives predicate upon current quality control and standardisation of protocols in a field where new developments are rapidly evolving, the current evidence on these issues is also reviewed.

4. Methods

Information on *T. cristatus* eDNA was obtained from the following sources:

(1) Existing literature held by the author.
(2) A search on Google Scholar and the Science Citation Index using Web of Science, using the terms '*Triturus cristatus*' or 'great crested newt' combined with 'eDNA', 'qPCR' and 'metabarcoding'.
(3) Natural Resources Wales Development mitigation reports in which eDNA had been used as a survey tool. Fourteen consultancy reports were provided along with a spreadsheet summarising some of the details within the reports. Because the actual number of ponds surveyed was sometimes not transparently stated in the report, or differed from that provided in the NRW spreadsheet, just the number of ponds surveyed for eDNA were included.

For (1) and (2) the title and abstract were read and if considered relevant to the review the paper was downloaded and scrutinised in detail. Any further relevant literature cited in existing review papers (i.e. Rees et al. 2014; Goldberg et al. 2016; Cristescu & Hebert 2018; Ficetola et al. 2019; Harper et al. 2019; Beng & Cortlett 2020; Fediajevaite et al. 2021; Bruce et al. 2021; Schenaker 2023; Sun et al. 2023; Iacaruso et al. 2025) that had not been obtained from the original searches were also examined for relevance.
For (3) the reports and spreadsheet were interrogated for information on (i) the number of ponds sampled for eDNA and the dates of sampling; (ii) the number of ponds that were assigned as eDNA positive within the reports; (iii) any supporting survey data from traditional surveys (i.e. torch counts, bottle trapping, netting or egg searches); (iv) the level of detail

provided in the reports (i.e. number of PCR replicates positive/negative per pond; tests for inhibition and degradation; sample quality assessment).

5. The WC1067 Great crested newt eDNA surveillance protocol

NRW currently accepts eDNA survey results providing they have strictly adhered to the WC1067 protocol and are collected between 15 April and 30 June (The use of environmental DNA test for Great crested newt licensing purposes).

This initial survey protocol was developed by Biggs et al. (2014) and was adopted by Defra and Natural England as the standard method required if eDNA results were to be accepted as a licensing requirement for survey in England. The same protocol was published for a wider audience by Biggs et al. (2015). The development of the protocol included (1) development and testing of a primer that would bind to and amplify *T. cristatus* DNA in water samples; (2) a comparison of the ability of eDNA to detect the species with traditional methods (i.e. torch counts, bottle trapping, egg searching) at 35 ponds with known histories from mid-April until June; (3) evaluation of eDNA to detect *T. cristatus* in 239 ponds sampled by volunteers both within and outside the known range of the species; and (4) a comparison of cost between eDNA and traditional surveys.

5.1. Pond sampling and laboratory analysis protocol

The recommended WC1067 protocol requires 20 x 30 ml samples to be collected at different locations around the pond margin. These are then pooled, and 6 x 15 ml subsamples taken and stored in separate tubes containing 35 ml of ethanol to preserve the DNA. Extraction of the DNA is then performed following concentration of the DNA from the six tubes via centrifugation and a modified Qiagen® DNeasy Blood and Tissue Kit protocol, and 12 replicate qPCRs run. The 'eDNA score' is based on the number of qPCR replicates out of 12 that successfully amplified. It was assumed that this score – expressed either as number out of 12 or a proportion – reflected the amount of eDNA contained in the sample, but even just one out of 12 amplifications could be taken as evidence of the presence of *T. cristatus* DNA, and by inference, the current or recent presence of the species. Appropriate controls were recommended to test for any contamination, inhibition or degradation of DNA during the process (see section 6).

5.2. Review of WC1067

Given that the original WC1067 protocol had been in use for nearly a decade without modification, Rees (2023) was commissioned by Natural England to review the protocol and provide recommendations to update it given the considerable progress made in eDNA sampling and analysis in recent years. As we recommend consultation of that report for

important suggested changes to the protocol, we provide only a summary of the key findings here along with other relevant research.

In line with other studies (e.g. Harper et al., 2019; Bruce et al. 2021), Rees (2023) concludes that collecting samples of water using filtration is an improvement on ethanol precipitation in that it will allow the sampling of larger volumes of water by concentrating DNA on a filter. Filtration is more efficient, reduces risk of contamination and avoids problematic issues concerning transport and disposal of ethanol. Comparisons of filtration with ethanol precipitation therefore show that filtration is frequently the more sensitive method for collecting eDNA from a water body, potentially collecting up to 5 times more DNA. That said, ponds are more problematic than flowing water to sample using filtration as they may contain more algae, detritus and suspended material that may quickly clog a filter (Rees 2023; Rees & Kane 2024). Sampling may take longer than storing in ethanol and a vacuum or peristaltic pump may be needed to suck water samples through a filter; this may make the protocol more cumbersome for volunteers. In a direct comparison of ethanol precipitation with filtration Rees & Kane (2024) found there was a high degree of consistency between the two methods in terms of eDNA detections, with 27 ponds yielding positive returns by both methods. That said, although filtration detected eDNA at five additional ponds where ethanol failed, ethanol also detected eDNA at five different ponds where filtration failed. There are also a variety of filter types available with different pore sizes and if results from different sites and studies are to be compared further standardisation of the equipment and materials is needed (Taugbøl et al. 2025). If ethanol precipitation is used then simple pre-filtering of the water sample may help reduce sediment that may affect DNA extraction and amplification.

Rees (2023) also highlights the problem of poor-quality samples that may be returned for lab analysis and recommends improved guidance for collecting water samples. eDNA may be unevenly distributed both vertically and horizontally within a pond, but sampling is usually confined to the accessible edges. Although gentle stirring of the water is recommended to increase the chances of eDNA capture, this should not be done to the extent that it also increases sediment and detritus within the water sample. A pre-analysis quality check could also include dip-testing of water quality to reduce the problem of calcium precipitation. In the laboratory, alternative DNA extraction kits may be worth considering, and recommended adjustments to the sampling protocol include reduction of centrifugation speeds, inclusion of additional protocols to reduce inhibition and degradation, and more precisely defined PCR cycling in amplification (see section 6).

Although it is therefore clear that recent developments in eDNA technology render the WC1067 protocol rather out of date, improving and revising the protocol so that it can meet statutory agency regulatory requirements may be a challenge. Industrial service providers and ecologists would need to invest in training to meet any new standards and this would proceed against a backdrop of ongoing scientific developments that may improve the protocol further. Equally, as even a well-defined protocol can be interpreted differently by different labs, there would need to be more rigorous proficiency testing to ensure there is consistency between labs (see section 10).

5.3. Interpretation of eDNA results in relation to imperfect detectability

The original WC1067 report compared eDNA to replicated torch counts, bottle trapping and egg searches at 35 ponds where *T. cristatus* was known to be present. Each pond was surveyed four times at approximately three-week intervals between mid-April and late June. Where eDNA successfully detected newts on 139 out of 140 survey visits – a 99.3% success rate – other survey methods were less successful: bottle trapping 76%; torch counts 75%; egg searches 44%. These findings correspond to earlier work on traditional survey methods that have shown that they suffer from imperfect detection, resulting in high levels of false negatives (and sometimes false positives; Sewell et al. 2010; Griffiths et al. 2015).

The WC1067 protocol assumes that the 'eDNA score' (i.e. the number of PCR replicates that amplify) is a reflection of the eDNA concentration in a pond. However, as with traditional methods, there is a possibility that eDNA may not be detected even if it is present. This can occur at either the pond sampling stage (i.e. Stage 1 - the eDNA is missed when taking the water sample) or at the laboratory stage (i.e. Stage 2 - the eDNA has been captured in the pond sample but fails to amplify and be detected in the lab). Either way, this will result in a 'false negative' result. Although the original WC1067 report indicated that false negatives were very low based on a sample of well-known *T. cristatus* ponds, subsequent field surveys have occasionally revealed negative eDNA results when a traditional method has indicated species presence at the time of sampling.

Occupancy modelling is an analytical tool that has been developed to estimate the rates of imperfect detection (i.e. false negatives and false positives) based on replicate analyses on a sample of sites (Ficetola et al. 2015). Such an occupancy model has now been developed specifically to estimate the rates of imperfect detection at the pond sampling and lab stages of analysis (Griffin et al. 2020; Diana et al. 2021). Buxton et al. (2022) applied this model to eDNA data collected and analysed by Natural England contractors using the WC1067 protocol from 4925 ponds across the country and explored the estimated rates of imperfect detection. The model estimated that the rate at which eDNA was 'missed' at the pond sampling stage was about 5.2% (with an upper range of 25.1% false negatives at some sites). At the laboratory stage, it is extremely unlikely that a pond sample that has captured eDNA will result in 0/12 PCRs amplifying. However, there is also a very low risk of obtaining a 'false positive' at the laboratory stage. This is likely to occur at a rate of about 2% for every PCR replicate. Consequently, although running 12 PCR replicates substantially reduces the chance of a false negative, it increases the chance of at least one PCR yielding a false positive to 24%. Exploration of the distribution of the number of PCRs amplifying revealed a bimodal pattern: there is a large peak where between 0-2 amplify, and a second peak where between 8-12 amplify. The initial peak includes the large number of ponds where *T. cristatus* were absent but also suggests that a small number of the ponds where just 1 or 2 PCRs amplified were probably false positives. Those ponds where 6 or more PCR replicates amplified were very likely 'true positives'. Although a result of 1/12 PCRs amplifying may reflect low concentrations of eDNA, there is also a risk of this being a false positive. Translating this into rates of pond occupancy in England, using 1/12 positive PCRs gives an occupancy rate of 30%; using a more stringent requirement of 2/12 positive PCRs gives an occupancy rate of 25%; and if 3/12 positive PCRs gives an occupancy rate of 22%.

Consequently, the interpretation of the eDNA results from a given pond requires scrutiny of the number of PCR replicates amplifying to balance the risk of concluding a false negative against the risk of a false positive as replication increases. Buxton, Matechou et al. (2021) suggest that accepting a minimum of 3/12 positive PCRs will reduce the risk of false positives and may be an improved threshold for assuming presence with a high degree of confidence. As Rees (2023) points out, however, if appropriate blanks are included as controls, and the PCR cycle is reduced to 50, it should be possible to identify if false positives are occurring and accept 1-2/12 as reliable. Likewise, if at least 6/12 positive PCRs are obtained then the result is very likely to be a 'true positive'. Equally, Buxton, Matechou et al. (2021) suggest a redistribution of sampling between stages 1 and 2 of the protocol. Two independent representative pond samples with six PCRs applied to each is preferable to one sample and 12 PCRs, as this will enable an assessment of whether within-pond variation in eDNA is occurring.

5.4. Conclusion - the WC1067 great crested newt surveillance protocol

The original WC1067 sampling protocol for eDNA can be improved upon by refinements to both field sampling and laboratory analysis protocols. This is likely to increase the sensitivity of eDNA as a method for detecting the presence of *T. cristatus* further. However, this greater sensitivity may render direct comparisons between previous surveys using WC10167 problematical.

Interpretation of eDNA results obtained using the WC1067 protocol requires careful scrutiny of the number of positive PCR replicates. Caution may be needed in accepting a return of 1-2/12 PCRs as a 'true positive', unless 'blank' controls also return as negative. False negatives are more likely to occur at the pond sampling stage than the laboratory analysis stage, and it is recommended that at least two independent – but representative – pond samples are taken at the same time that cover as much of the pond area as possible and that these are subject to 6 PCR replicates each.

6. Persistence, inhibition, degradation and contamination of eDNA

As the detection of eDNA in a pond indicates the current – or recent – presence of the species, it is important to understand how long it may persist. DNA can be degraded by a variety of environmental factors, such as temperature, ultraviolet light or chemical or microbial decay. It can also become undetectable through binding with sediment or other particulate matter, or it can be transported or diluted out of the system by rainfall or water flow (Pilliod et al., 2014; Rees et al., 2014; Harper et al., 2019; Beng & Cortlett 2020; Bruce et al., 2021). Thomsen et al. (2012) observed eDNA concentrations to decline after newts were removed from experimental tanks and became undetectable after 1-2 weeks. Buxton et al. (2017a) showed that water sampled from high density newt ponds initially displayed

high detectability of eDNA but that this declined to 10-22% after 15 days. However, the persistence of eDNA depended on the substrate, with detectability lower in topsoil than in clay, sand or controls (no substrate). In sum, although decay rates may be variable, eDNA may remain detectable in ponds for a few days after newts are removed.

Environmental DNA may become undetectable by laboratory assays due to inhibition or degradation. Inhibition of the PCR amplification occurs when the sample contains matter that interferes with this process. This can be tested for by including an appropriate control in the PCR process (i.e. adding a positive control) – if the PCR fails to amplify a known quantity of DNA that has been added then the sample can be considered as inhibited. Rees (2023) stated that 8% of samples sent to RSK ADAS for testing displayed inhibition, and discusses how this can be mitigated, including an additional stage using PCR inhibition removal kits. When there is a risk of high concentrations of inhibitors, digital droplet PCR (ddPCR) may outperform real-time PCR in the detection of fish (Doi et al. 2015). Degradation occurs if the eDNA collected is already in a degraded state, or degradation occurs after the collection process. Again, including an appropriate control that comprises a known quantity of DNA within the preservative that is used in the collecting kits can test for any degradation due to preservation failure, and is recommended for inclusion after inhibition tests (Rees 2023). Such tests of inhibition and degradation should be included in all reporting of eDNA survey results along with any information to mitigate their impacts. Bruce et al. (2021) provide a user-friendly guide to the recommended controls needed to test for inhibition and degradation.

A further source of error is when samples become contaminated by target DNA either in the field or laboratory. Although ensuring rigorous protocols are followed should reduce this risk, including negative controls (i.e. samples of water that are known to be DNA-free) should go some way to identifying whether contamination has occurred in the workflow (Hutchins et al. 2022).

6.1. Conclusion – persistence, inhibition, degradation and contamination

We recommend that the tabulated summary provided by Bruce et al. (2021) that describes the different types of controls needed along the workflow is consulted so that cases of inhibition, degradation and contamination can be reliably identified and reported.

7. Seasonal variation in the concentration of eDNA

The original WC1067 protocol was based on samples taken between 15 April and 30 June, a period assumed to embrace the main breeding period for *T. cristatus* and when adults were most likely to present in ponds. Consequently, the acceptance of eDNA records obtained under licence from Natural England stipulated that samples must be taken during

this period, and NRW have also adopted this sampling timeframe. Subsequent work has shown that it is quite possible to detect *T. cristatus* outside of this survey window and this has led to calls for the licensed survey window to be extended (Gorman et al., 2020). For example, Rees et al. (2017) showed that it was possible to detect *T. cristatus* in some ponds from March to February (based on at least 1 out 12 PCRs amplifying). However, the eDNA scores fell from a median of 9/12 during the period when adults were known to be present to a median of 3/12 from September-February when adults were not observable. Equally, *T. cristatus* has been detected outside the main breeding period in studies on the continent (e.g. Charvoz et al. 2021; Strand et al. 2022).

Buxton et al. (2017b) examined the seasonal variation of *T. cristatus* in more detail between February and October in a series of small, replicated artificial ponds that had been naturally colonized by the species. This enabled the concentration of eDNA to be tracked across the season in relation to trends in adult population size, egg laying and larval production. The study ponds were known to contain a very high density of adults during the main breeding period, but with some individuals present outside this period including the winter. Environmental DNA was detectable in the ponds throughout the period from February to October but displayed two conspicuous peaks in concentration. The first peak corresponded to early June when adults were losing body mass, probably through egg-laying and releasing spermatophores that contain DNA. The second peak occurred between mid-July and mid-August and reflected the DNA shed by hatching and growing larvae. When larvae started metamorphosing and leaving the ponds the concentrations of eDNA dropped dramatically. Superimposed on these trends was a temperature influence on eDNA concentration, although neither rainfall nor UV seemed to have an impact. In ectotherms higher temperatures may result in increased metabolism and activity, resulting in higher DNA shedding rates. This study shows that in this high-density population it is possible to detect eDNA outside the recommended survey window of April-late June, but as eDNA concentrations may drop outside the breeding period it may be undetectable in some ponds within a landscape. Indeed, should reproduction in a given year be eliminated by, for example, fungal infection of eggs or invertebrate predation, then eDNA may not be detectable outside the recommended survey window even if adults are present earlier in the year. This suggests that the detection of eDNA after late June may indicate the presence of *T. cristatus* but non-detection does not necessarily reflect absence of a breeding population.

A further survey of *T. cristatus* eDNA in both water and sediment from a wider sample of ponds showed that the probability of detection of eDNA was highest in the spring and summer and then fell during the autumn to very low levels in January (Buxton et al. 2018). These issues were further explored on a wider sample of more natural ponds surveyed between April and October by Harper et al. (2025). They found that eDNA detection was possible up to August but decreased in September and October. As flagged by Buxton, Foster et al. (2021) they conclude that out-of-season non-detections deserve cautious interpretation. Positive eDNA detections up to August may be a reliable indicator of species presence, but non-detections up to and beyond this time cannot be assumed to be an indicator of species absence.

7.1. Optimising the survey window for eDNA sampling

Given that the seasonal abundance of eDNA in pond water will reflect eDNA release by different life stages at different times, the timing of sampling ideally requires an understanding of the dynamics of adults, eggs and larvae during the year. These dynamics are regulated by hydroperiod, disease, competition and predation. For example, should a pond with a high density of adults dry in May, it will force adults to leave the pond, and any eggs and larvae will not survive. Should that pond be recharged by unseasonal rains in the summer, then an eDNA sample – or for that matter, any other survey – from that time will be negative. Likewise, in some ponds in some years all eggs and/or larvae may be lost to disease, competition or predation from invertebrate or vertebrate predators. In such cases, even if a pond contains a reproductive population of newts, it is likely to yield a negative eDNA result if a sample is taken at a time after adults have left and eggs and larvae have been eliminated. *T. cristatus* metapopulation dynamics operate on a 'source' (= ponds that have successful reproduction) and 'sink' (= ponds where there is reproductive failure). However, sources may become sinks in different years and vice versa (Griffiths, 2004). This pattern has been confirmed by the national PondNet survey in England, which showed that only 14% of ponds that were positive at some point over a decade were positive in all years (Ewald and Carter 2025). These issues need to be considered when taking or interpreting the results of eDNA samples taken as a time series.

Preliminary analysis of data from the NRW 2023 online database, covering 58 surveyed sites, showed peak counts of adult *T. cristatus* distributed as follows:

- 25% of sites recorded peak numbers between March and 14 April,
- 60% between 15 April and 14 May,
- 15% after 15 May, with only one site recording a peak in June.

The conclusion was that 'peak breeding activity in Wales typically occurs between mid-April and mid-May'. Acknowledging that this may vary between years and sites (e.g. at an upland pond in mid-Wales Griffiths & Mylotte (1987) observed a peak in July with adults present through to September), this survey window broadly reflects the period when adult newts reach their peak across large areas of Britain. As observed by Buxton et al. (2017b), however, peaks in eDNA concentrations may not simply be related to peaks in adult abundance. Indeed, a major peak in eDNA occurs in June when newts are laying eggs and larvae are developing, which was later than when the adult numbers peaked. Given this pattern, restricting the period over which eDNA can be collected to between 15 April and 30 May risks missing (1) a period during which adults are shedding most eDNA into the water; and (2) a period in July-August when larvae are also shedding eDNA into the water. This suggests it may be wiser to maintain the sampling period at least until the end of June. Indeed, does this not also suggest that the sampling window could be extended until August, as advocated by Rees et al. (2017) and Gorman et al. (2020)? Although sampling in August could detect eDNA released by larvae, if reproductive effort fails in that year then it would likely yield a negative result for a pond that contains breeding adults. We therefore recommend that the current survey window for using eDNA to determine likely presence-

absence is maintained as 15-April-30 June. On the basis that larvae may be present at some – but not all – *T. cristatus* breeding ponds after 30 June, samples after that date that prove positive may be acceptable, but negative samples may be an unreliable indicator of true absence. As suggested by Buxton et al. (2017b) and Harper et al. (2025) we therefore recommend that water bodies can be surveyed using eDNA until August for presence, but not for absence. Even though *T. cristatus* may be detectable in ponds after August, the probability of detection during the autumn falls substantially. Put simply, if eDNA samples were taken in the autumn when *T. cristatus* was present, they would likely miss the species on 50% of occasions; this would drop to just 20% of occasions in the winter months due to the scarcity of eDNA at that time (Buxton, Foster et al., 2021). In addition, an understanding of the dynamics of hydroperiod, life history and competition and predation at individual ponds may assist in the interpretation of eDNA results.

7.2. Conclusion – seasonal variation in the concentration of eDNA

High concentrations of eDNA during the summer months (July-August) are likely due to the presence of developing eggs and larvae rather than adults. As reproduction often fails due to egg infections or predation of larvae, negative eDNA samples taken at this time may reflect breeding failure rather than the absence of a breeding population. Negative eDNA samples taken during the summer months therefore need interpreting with caution. Although low numbers of larvae and adults may be present in ponds during the autumn and winter months eDNA concentrations – and hence the probability of detection of eDNA – will be much lower. Without other data collected during the main breeding period, negative eDNA samples collected after 30 June should not be interpreted as absence of *T. cristatus*.

8. Relationship between eDNA and species abundance

Because concentrations in a pond are a balance between eDNA production and eDNA removal, with both being influenced by environmental factors, studies to establish a relationship between eDNA and abundance have yielded mixed results (Yates et al. 2018; Beng & Cortlett 2020). The pioneering work by Thomsen et al. (2012) did report a positive relationship between eDNA concentration and *T. cristatus* density in both natural and controlled sampling units. Biggs et al. (2014; 2015) found a weak relationship between eDNA score and number of newts counted by torch or bottle trapping. Although low eDNA scores were invariably associated with low counts, high eDNA scores (i.e. 12/12 positive PCR replicates) were found for ponds that had both low and high counts (i.e. counts varying between 0 and over 60). Overall, recent reviews have emphasized the difficulty in establishing a relationship between eDNA and measures of species abundance (Ficetola et al. 2019; Beng & Cortlett, 2020), and positive relationships may be commoner in contrived laboratory situations than they are in field studies (Yates et al. 2018). Equally, this pattern may reflect more the unreliability of simple counts as a reflection of population size and/or eDNA biomass rather than the unreliability of eDNA to detect newts. Indeed, the widely used system to allocate newt populations to a class size of ‘Low’, ‘Good’ or ‘Exceptional’ based

on unstandardized simple counts is more a measure of newt detectability than newt 'abundance' (Griffiths et al. 2015), so any attempt to seek a relationship with eDNA concentrations will be fraught with caveats. Theoretically, at any point in time eDNA concentration may reflect the biomass of the species – adults, gametes or larvae – but given the range of environmental factors that influence the production and removal of eDNA in a system, the relationship between the two will be site and time specific. At present then, attempts to extrapolate measures of *T. cristatus* eDNA in a pond to population indices should be avoided.

8.1. Conclusion – relationship between eDNA and species abundance

At present there is little evidence that there are consistent relationships between eDNA concentration and abundance. This may be down to high levels of uncertainty in abundance measures as well as variable eDNA production and removal rates.

9. Cost-effectiveness

Because of high levels of variation in detectability using traditional methods (i.e. torch counts, egg searches and bottle trapping), four repeat surveys are needed to achieve the same level of detectability as eDNA (Biggs et al. 2014). Moreover, bottle trapping requires two visits to each pond per survey. On this basis – and the assumption that eDNA sample can be taken by volunteers while traditional methods are undertaken by professionals – eDNA surveys worked out as 6-10 times cheaper than traditional surveys. For Wales, Biggs et al. (2014) estimated that the total costs to survey 600 ponds in 300 x 1 km grid squares would cost £2,714,000 if traditional surveys were carried out by professionals and £411,00 for an eDNA survey carried out by a team comprising half professionals and half volunteers. Rees (2023) describes various ways that the current WC1067 protocol may be made more cost-effective, such as using alternative kits, non-sterile equipment, cheaper centrifuges and extending the life of kits from two weeks to three months. Likewise, a wider metanalysis revealed that eDNA surveys are cheaper, more sensitive and able to detect more species than traditional surveys and are particularly suited for amphibians (Fediajevaite et al. 2021). In Australia, Smart et al. (2016) compared bottle trapping to eDNA surveys for non-native smooth newts (*Lissotriton vulgaris*). As in the UK, eDNA resulted in much higher detection rates than bottle trapping, but the cost-effectiveness of the two methods was similar if primer/probe development and sample processing was costly and included in budgeting. If the WC1067 protocol is modified to include water sample filtration the costs involved with ethanol disposal would disappear, but the method may require professional – rather than volunteer – expertise and require more time to obtain a sample from a pond. As Biggs et al. (2014) point out, although using volunteers reduces costs, volunteers prefer to travel to sites close to home, may be unwilling to visit sites where land ownership is not known, and prefer opportunities where they may see amphibians. Consequently, an appropriate level of professional backup and logistical support needs to be in place even when surveys are carried out by volunteers.

9.1. Conclusion – cost-effectiveness

As they require fewer site visits and do not require as much professional training, eDNA surveys are generally more cost-effective in terms of data quality and quantity than traditional surveys. Modifications to the WC1067 protocol may improve the cost-effectiveness further, although there could be increases in costs if water filtration is incorporated into the protocol. Cost-effectiveness may also hinge on the balance between professional and volunteer input.

10. Quality control, proficiency testing and standardisation

Comparison of different eDNA results in time and space is very difficult unless there is confidence that different workers are applying exactly the same protocol and quality control procedures in both the field and the lab (Goldberg et al. 2016; Cristescu & Hebert 2018; Thalinger et al. 2021; Theroux et al. 2025). Since 2017 Fapas (the Fera proficiency testing division) has offered an annual great crested newt eDNA proficiency testing service to laboratories. The scheme scrutinizes the processing capabilities and workflows of participating labs and their ability to identify eDNA at different concentrations. For Natural England to accept the results of eDNA testing laboratories must participate in the proficiency testing, which is a closed system operating by invitation (Rees & Gough 2018; Trujillo-Gonzalez et al. 2021; Rees 2023). This has shown that there may be variation between laboratories in the accuracy of analyses. A comparison between seven anonymised labs provided with the same samples of DNA revealed that the number of amplifying PCR replicates they returned varied (Rees & Gough 2018). Equally, Rees (2023) points out that over seven years of testing since 2017, 100% identification of eDNA has only been achieved in one year (2021), with overall 'satisfactory performance' in other years ranging from 84-99%. Failures can be down to low rates of false negatives, false positives, contamination and sample misidentification. As there is no baseline minimum standard, the proficiency test provides an opportunity for service providers to review their own protocols and workflows and make any adjustments they deem necessary. As such information is commercially sensitive the performances of individual labs are anonymized.

Despite proficiency testing then, there is some inevitable variation between laboratories providing accurate eDNA results. Rees (2023) suggests that further clarity is needed in how service providers can be retested if they implement improvements after failing to achieve a minimal standard. Based on the MIBBI project ((http://www.mibbi.org/) Goldberg et al. (2016) provide guidelines on the minimum information required for sampling, analysis and reporting of eDNA studies and Thalinger et al. (2021) provide a validation scale for assays. The DNAqua.net consortium is also developing tools that can guide the standardisation of eDNA freshwater sampling procedures (DNAqua-Net | COST Action CA15219). Theroux et al. (2025) provide a summary of the various efforts around the world to try and standardise eDNA methods.

10.1. Conclusion – quality control, proficiency testing and standardisation

Although there is inevitably some variation in eDNA results returned by different service providers, with careful reporting and interpretation this should not usually lead to differences in conclusions. As far the WC1067 protocol is concerned, it would be helpful if reports can contain standardised information about the outputs; i.e. any issues collecting the samples; any pre-testing sample quality control assessment; the number of PCR replicates that were positive, negative or inconclusive; the number of PCR replicates that were inhibited or degraded.

11. Metabarcoding

Since the WC1067 protocol was developed and delivered, metabarcoding has largely superseded single species eDNA surveys for biodiversity assessment. Harper et al. (2018) found that qPCR was marginally more sensitive than metabarcoding in detecting *T. cristatus* eDNA, although this depended on where the thresholds for 'positive' were set in both analyses. Reanalysing the Harper et al. (2018) data using different models, McColl-Gausden et al. (2023) also concluded that generally, qPCR was more sensitive than metabarcoding in detecting *T. cristatus*, but the relative sensitivity of the two methods did depend on the study design and thresholds used to interpret the results. In a direct comparison of the two methods Rees & Kane (2024) found that qPCR was more sensitive at detecting low concentrations of DNA than metabarcoding. However, such caveats need to be set against the benefits of the extra ecological information on freshwater systems that can be generated within a metabarcoding analysis. As with single species qPCR protocols, low rates of false negatives and false positives may occur in metabarcoding studies. At the laboratory analysis stage, these may be down to rare DNA sequences being missed in the bioinformatic pipeline, primer biases, index switching or contamination (Deiner et al. 2017; Cristescu & Hebert 2018; McColl-Gausden et al. 2023).

However, as with the qPCR method, metabarcoding may detect species where visual surveys have failed (Charvoz et al. 2021; Svenningsen et al. 2022). Although the number of DNA barcode sequence reads allocated to a species in a sample may reflect the amount of DNA – and intuitively – the abundance of the species, as with qPCR there are many environmental factors that can affect DNA concentrations so such results need to be treated with caution (Ficetola et al. 2019). Models are being developed to account for changes in eDNA biomass within species across different sites, accounting for imperfect detection at the different stages of the metabarcoding workflow and the potential covariates that may explain such variation (Diana et al. 2025).

11.1. Conclusion – metabarcoding

Compared to the single species qPCR WC1067, metabarcoding can provide more detailed information on multiple species and ecological processes. It may have less sensitivity than

qPCR when very low concentrations of *T. cristatus* DNA are present, but direct comparisons between the methods depend on the study design and thresholds used in interpreting the data. Equally, direct comparisons between metabarcoding outputs and historical data or data collected using other methods need to be performed with caution.

12. Interpretation of eDNA results from NRW reports

The level of detail provided in the NRW consultancy reports was variable. Not all target ponds were possible to survey because of accessibility, permission withdrawal or pond desiccation, and in some cases it was not possible to take representative eDNA samples from around the shoreline. One report did not contain a report of eDNA surveys. In total, 97 ponds were surveyed for eDNA of which 15 were assigned as positive for *T. cristatus*. Two reports did not contain explicit dates for the sampling day, and one report contained data taken in July which is outside the accepted survey window, although the conclusions were appropriately qualified. All other eDNA samples were taken between 29 March (also outside the WC1067 survey window) and 30 June. Eight reports contained results of eDNA only, with no accompanying traditional surveys. Traditional surveys were usually carried out if a population size class assessment was deemed necessary following a positive eDNA result. As a result, there was only one report where eDNA samples were taken at the same time as traditional surveys. There was only one case where a follow-up visual survey did not correspond with eDNA results. This involved the observation of a single juvenile *T. cristatus* at a pond where the eDNA test had previously proved negative. As the eDNA sample was not taken simultaneously with the follow-up survey it is possible that this individual newt entered the pond after the eDNA sample had been taken. Alternatively – and as smooth newts were also observed in the same pond – this individual may have been a misidentification. Direct comparisons of contemporary records with historical data are not a valid way to calibrate a survey method because of the natural temporal fluctuations in *T. cristatus* populations and the fact that not all ponds are occupied every year (see section 7.1).

Eight out of the fourteen reports provided a detailed breakdown of eDNA result from each pond and described the pre-analysis sample assessment; the number of replicates out of 12 that amplified; the number that passed inhibition and degradation tests (in fact none were reported to have failed these). One report referred to a Table that was missing, four reports simply described the ponds as either positive or negative, and one did not include any eDNA findings.

12.1. Conclusion – interpretation of eDNA results from NRW reports

To improve the interpretation of eDNA results, NRW could request more standardisation and consistency in reporting (see conclusion 11.1).

13. Recommendations

- NRW should consider adopting an updated eDNA sampling protocol to that provided by WC1067. Liaison with Natural England and NatureScot over the adoption of the recommendations provided by Rees (2023) would provide more consistency across the agencies.

- Replacing ethanol precipitation with filtration will allow a greater volume of water to be sampled and may improve the sensitivity of eDNA detection.

- The guidance provided by Bruce et al. (2021) should be consulted for appropriate controls for contamination, degradation and inhibition.

- Unless additional information on multispecies is required, qPCR is currently recommended over metabarcoding for detecting *T. cristatus* eDNA.

- The recommended sampling window should remain as 15 April until 30 June.

- It is recommended that ecological reports consider/make reference to potential pond functionality (as informed by Section 7.1)

- Although it would increase the sampling effort, two independent pond samples collected at the same time and preserved in two sets of 6 x 15 ml tubes and which are subsequently subjected to 6 x PCR replicates each would be preferable to a single pond sample subjected to 12 PCR replicates.

- Shortening the sampling window is not recommended as it may miss eDNA released through the development and growth of larvae.

- Samples that are positive for eDNA that are taken after 30 June are acceptable as evidence for the presence of *T. cristatus*.

- Samples that are negative for eDNA that are taken after 30 June are not acceptable as evidence for the absence of *T. cristatus* unless there is supporting evidence that no stages of the life cycle were present during the main sampling window of 15 April-30 June.

- Where there is any uncertainty over the interpretation of eDNA results or high risks are attached to any potentially uncertain results, corroboration of the results should be obtained by repeat sampling and/or analysed by a different independent service provider.

- eDNA results cannot currently be used to reliably infer measures of abundance.

- eDNA results should only be accepted if provided by laboratories participating in the Fapas proficiency testing scheme.

- When reporting results from eDNA surveys, practitioners must include information on pre-analysis quality control of the samples; descriptions of the controls used to test for contamination, degradation and contamination and their results; the number of PCR replicates that returned positive and negative; any other relevant methodological details (e.g. use of any pre-filtering; use of inhibition removal kits; dip-tests for calcium precipitation).

14. References

Beng, KC, Corlett, RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation 29, 2089-2121.

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P, Williams P, Dunn F. 2014. Analytical and methodological development for improved surveillance of the Great Crested Newt. Defra Project WC1067. Freshwater Habitats Trust: Oxford.

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P, Williams P, Dunn F. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biological Conservation 183, 19-28.

Bruce K, Blackman RC, Bourlat SJ, Hellström M, Bakker J, Bista I, Bohmann K, Bouchez A, Brys R, Clark K, Elbrecht V, Fazi S, Fonseca VG, Hänfling B, Leese F, Mächler E, Mahon AR, Meissner K, Panksep K, Pawlowski J, Yáñez PLS, Seymour M, Thalinger B, Valentini A, Woodcock P, Traugott M, Vasselon V, Deiner K. 2021. A practical guide to DNA-based methods for biodiversity assessment. Sofia, Bulgaria: Pensoft.

Buxton AS, Groombridge, JJ, Griffiths, RA. 2017a. Is the detection of aquatic environmental DNA influenced by substrate type? PLoS ONE 12(8): e0183371.

Buxton, AS, Groombridge, JJ, Zakaria, NB, Griffiths, RA. 2017b. Seasonal variation in environmental DNA in relation to population size and environmental factors. Scientific Reports 7:46294.

Buxton AS, Groombridge, JJ, Griffiths RA. 2018. Seasonal variation in environmental DNA detection in sediment and water samples. PLoSONE 13(1): e0191737.

Buxton AS, Matechou E, Griffin J, Diana A, Griffiths RA. 2021. Optimising sampling and analysis protocols in environmental DNA studies. Scientific Reports 11:11637.

Buxton AS, Foster J, Griffiths RA. 2021. The importance of considering detection probability and species ecology in ecological surveys: a response to Gorman et al., extended season environmental DNA surveys for great crested newts. In Practice 111, 55-58.

Buxton AS, Diana A, Matechou E, Griffin J, Griffiths RA. 2022. Reliability of environmental DNA surveys to detect pond occupancy by newts at a national scale. Scientific Reports 12:1295.

Charvoz L, Apothéloz-Perret-Gentil L, Reo E, Thiébaud J, Pawlowski J. 2021. Monitoring newt communities in urban area using eDNA metabarcoding. PeerJ 10.7717/peerj.12357.

Cristescu ME, Hebert PDN 2018. Uses and misuses of environmental DNA in biodiversity science and conservation. Annual Review of Ecology, Evolution and Systematics 49, 209-230.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N, Pfrender ME, Bernatchez L. 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular Ecology 26, 5872–5895.

Diana A, Matechou E, Griffin, JE, Buxton, AS, Griffiths, RA. 2021. An Rshiny app for modelling environmental DNA data: accounting for false positive and false negative observation error. Ecography 44, 1838-1844.

Diana A, Matechou E, Griffin J, Yu DW, Luo M, Tosa M, Bush, A, Griffiths, RA. 2025. eDNAPlus: A unifying modelling framework for DNA-based biodiversity monitoring. Journal of the American Statistical Association 120, 120-134.

Doi H, Takahara T, Minamoto T, Matsuhashi S, Uchii K, Yamanaka H. 2015. Droplet digital Polymerase Chain Reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. Environmental Science & Technology 49, 5601-5608.

Ewald N, Carter A. 2025. PondNet: Monitoring background trends in great crested newt conservation status in England: Survey Results 2015-2024. Freshwater Habitats Trust, Oxford

Fediajevaite J**,** Priestly V, Arnold, R, Savolainen V**.** 2021. Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. Ecology and Evolution 11, 4803-4815.

Ficetola GF, Manenti R, Taberlet P. 2019. Environmental DNA and metabarcoding for the study of amphibians and reptiles: species distribution, the microbiome, and much more. Amphibia-Reptilia 40, 129-148.

Ficetola GF, Pansu J, Bonin A, Coissac E, Giguet-Covex C, De Barba M, Gielly L, Martins Lopes C, Boyer F, Pompanon F, Rayé J, Taberlet P. 2015. Replication levels, false presences, and the estimation of presence/absence from eDNA metabarcoding data. Molecular Ecology Resources 15, 543-556.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE, Taberlet, P. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7, 1299-1307.

Gorman L, Nisbet S, Wansbury C. 2020. Extended season environmental DNA surveys for great crested newts. In Practice 110, 50-51.

Griffin JE, Matechou E, Buxton AS, Bormpoudakis D, Griffiths RA. 2020. Modelling environmental DNA data; Bayesian variable selection accounting for false positive and false negative errors. Journal of the Royal Statistical Society Series C 69, 377-392.

Griffiths RA. 2004. Great crested newts in Europe: the effects of metapopulation structure and juvenile dispersal on population persistence. In: Ackakaya HR, Burgman MA, Kindvall, O. (eds), Species Conservation and Management: Case Studies. Oxford University Press, New York, pp. 281–291.

Griffiths RA, Mylotte, VJ. 1987. Microhabitat selection and feeding relations of smooth and warty newts, *Triturus vulgaris* and *T. cristatus*, at an upland pond in mid-Wales. Holarctic Ecology 10, 1-7.

Griffiths, RA, Foster, J, Wilkinson, JW, Sewell, D. 2015. Science, statistics and surveys: a herpetological perspective. Journal of Applied Ecology 52, 1413-1417.

Harper LR, Lawson Handley L, Hahn C, Boonham N, Rees HC, Gough KC, Lewis E, Adams IP, Brotherto, P, Phillips S, Hänfling B. 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). Ecology and Evolution 8, 6330‐6441.

Harper LR, Buxton AS, Rees HC, Bruce K, Brys R, Halfmaerten D, Read DS, Watson HV, Sayer CD, Jones EP, Priestley V, Mächler E, Múrria C, Garcés-Pastor S, Medupin C, Burgess K, Benson G, Boonham N, Griffiths RA, Lawson Handley L, Hänfling B. 2019. Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. Hydrobiologia 826, 25-41.

Harper LR, Harper KJ, Platts S, Irwin R, Bennett M, Jones B, Taylor O, Plant L, Eccleshall, D, Briscoe A, Gorman L, Stanhope K, Egeter, B. 2025. Extending sampling approaches for great crested newt (*Triturus cristatus*) eDNA monitoring. Ecological Solutions and Evidence DOI: 10.1002/2688-8319.70160.

Hutchins PR, Simantel, LN, Sepulveda, AJ. 2022. Time to get real with qPCR controls: The frequency of sample contamination and the informative power of negative controls in environmental DNA studies. Molecular Ecology Resources 22, 1319-1329.

Iacaruso NJ, Reves OP, Merkelz SP, Waldrep CL, Davis, MA. 2025. A systematic review evaluating the performance of eDNA methods relative to conventional methods for biodiversity monitoring. Ecography e07952.

McColl-Gausden EF, Weeks AR, Coleman R, Song S, Tingley R. 2023. Using hierarchical models to compare the sensitivity of metabarcoding and qPCR for eDNA detection. Ecological Informatics 75, 102072.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources 14, 109-116.

Rees, HC. 2023. An evidence review for great crested newt eDNA monitoring protocols. Natural England Commissioned Report NECR476.

Rees H, Gough, K. 2018. Great crested newt eDNA laboratory quality systems, proficiency testing and interpretation of results. In Practice 99, 24-27.

Rees, HC, Kane SD. 2024. GCN sample analysis via single species assay and metabarcoding, 2024. NECR534. Natural England.

Rees, HC, Bishop K, Middleditch DJ, Patmore JRM, Maddison BC, Gough KC. 2014. The application of eDNA for monitoring of the Great Crested Newt in the UK. Ecology and Evolution 21, 4023-4032.

Rees HC, Maddison BC, Middleditch DM, Patmore JRM, Gough, KC. 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. Journal of Applied Ecology 51,1450-1459.

Rees HC, Baker, CA, Gardner DS, Maddison BC, Gough, KC. 2017. The detection of great crested newts year round via environmental DNA analysis. BMC Research Notes 10:327.

Schenekar, T. 2023. The current state of eDNA research in freshwater ecosystems: are we shifting from the developmental phase to standard application in biomonitoring? Hydrobiologia 850, 1263-1282.

Sewell D, Beebee TJC, Griffiths RA. 2010. Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. Biological Conservation 143, 2102–2110.

Smart AS, Weeks AR, Rooyen AR, Moore A, McCarthy MA, Tingley R. 2016. Assessing the cost-efficiency of environmental DNA sampling. Methods in Ecology and Evolution 7, 1291-1298.

Strand LÅ, Måseide HK, Gaupeland S, Johansen BS, Slettan A. (2022). Great crested newt *Triturus cristatus*, smooth newt *Lissotriton vulgaris*, and other amphibians in an acidified area of southern Norway surveyed using eDNA and other methods. Herpetological Bulletin 161, 5-11.

Sun X, Guo N, Gao J, Xiao N. 2024. Using eDNA to survey amphibians: Methods, applications, and challenges. Biotechnology Bioengineering 121, 456-471.

Svenningsen AKN, Pertoldi C, Bruhn D. 2022. eDNA metabarcoding benchmarked towards conventional survey methods in amphibian monitoring. Animals 12, 763.

Taugbøl A, Fossøy F, Dervo BK. 2025. Detecting environmental DNA from crested and smooth newts - not as straight forward as filtering a drop of water. Aquatic Conservation: Marine and Freshwater Ecosystems 35, e70106.

Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS, Bruce, K. 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA 3, 823-836.

Theroux S, Sepulveda A, Abbott C., Gold Z, Watts AW, Hunter ME, Klymus KE, Hirsh SL, Craine J., Jones DN, Brown RJ. 2025. What is eDNA method standardisation and why do we need it? Metabarcoding and Metagenomics 13(9), e132076.

Thomsen PF, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP, Orlando L, Willerslev E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology 21, 2565-2573.

Trujillo-González A, Villacorta-Rath C, White NE, Furlan EM, Sykes M, Grossel G, Divi UK, Gleeson D. 2021. Considerations for future environmental DNA accreditation and proficiency testing schemes. Environmental DNA 3, 1049-1058.

Yates MC, Fraser DJ, Derry AM. 2018. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. Environmental DNA 1, 5-13.