Welsh Government

M4 Corridor around Newport

August 2017 Environmental Statement Supplement Appendix ESS5 2.5

Great Crested Newt eDNA Survey

M4CaN-DJV-EBD-ZG_GEN-AX-EN-0056

At Issue | August 2017

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Welsh Government

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Newport Docks – South: Great Crested Newt eDNA Survey, 2017

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Contents

Summary

S.1 RPS has undertaken a great crested newt survey of an area of land on the southern part of the Newport Docks in Newport, South Wales. The survey area is shown on Figure 1.

S.2 The survey area included land that would be indirectly affected by the proposed M4 Corridor around Newport (M4CaN) Scheme. Should the Orders for the Scheme be confirmed, buildings located elsewhere on the docks would be relocated to the survey area in order to enable the motorway construction to be carried out.

S.3 The survey method was based on that described in Biggs, *et al.* (2014). The survey comprised collecting water samples from waterbodies of potential value to great crested newts for laboratory analysis for great crested newt environmental DNA (eDNA).

S.4 The survey visit was undertaken on the 29 June 2018, a suitable date for this type of survey.

S.5 Water samples were collected from two ponds and one reen. All other waterbodies in the survey area were dry at the time of the survey visit.

S.6 Results of the laboratory analysis reported no positive results for great crested newt eDNA.

S.7 Results of a Phase 1 habitat survey of the area undertaken by RPS in June 2017 (Appendix ESS5 2.3) reported waterbodies to be of low potential value to great crested newts due to the limited amount of aquatic/semi-aquatic vegetation; the likelihood of waterbodies becoming dry during the breeding season; and the isolated nature of the survey area, which was surrounded by the Rivers Usk and Ebbw to the east, west and south, and built-up areas to the north, and separated from known populations of great crested newts by the River Usk (Appendices 10.6 and 10.22 to the March 2016 M4CaN Environmental Statement, Welsh Government 2016).

S.8 Taking the above into account, great crested newts are considered to be absent from the survey area and no further survey work is recommended.

1 Introduction

1.1.1 RPS was commissioned to undertake a great crested newt eDNA survey of waterbodies on an area of land in the Newport Docks in Newport, South Wales. The survey area is shown on Figure 1.

1.1.2 The survey area included land that would be affected by the proposed M4 Corridor around Newport Scheme (M4CaN). Should the Orders for the Scheme be confirmed, buildings located elsewhere on the docks would be relocated to the survey area in order to enable the motorway construction to be carried out.

1.1.3 The objective of the survey was to determine the presence or likely absence of great crested newts in surveyed waterbodies in order to inform an assessment of the likely effects of the M4CaN Scheme with regard to great crested newts and any mitigation that might be required.

1.1.4 This report describes the survey method used (Section 2) and the results of the survey (Section 3). A discussion of the survey results is provided in Section 4.

2 Survey Method

2.1 Introduction

2.1.1 The survey visit was undertaken on the 29 June 2017, a suitable time for this type of survey (Annex A).

2.1.2 The survey method followed that described in Biggs *et al*, 2014.

Field protocol

2.1.3 The survey team was trained in the eDNA surveying technique, as described by Biggs *et al.* (2014), by a licensed senior ecologist prior to collecting water samples.

2.1.4 Water samples were collected from two ponds (Ponds 1 and 2) and a reen, as shown on Figure 1. The three waterbodies were identified during a Phase 1 habitat survey of the area (Appendix ESS5 2.3) as being of some potential value to great crested newts. At the time of the survey visit, all other waterbodies in the survey area were dry.

2.1.5 Water samples were collected using sampling kits supplied by SureScreen Scientifics laboratory, which was responsible for analysing the samples.

2.1.6 Surveyors collected 30 millilitre water samples from twenty locations along the margins of each waterbody using a sterile ladle. Surveyors collected the sample from the bank edge and did not enter the water. Where access allowed, the samples were collected from points evenly spaced along the bank of each waterbody.

2.1.7 When collecting the samples, surveyors used a sterile ladle to gently agitate the water and mix the water column, whilst taking care not to disturb and collect any sediment. The twenty samples collected from each waterbody were then emptied into a sterile plastic bag and homogenised by gently shaking the bag to ensure eDNA was evenly mixed through the sample.

2.1.8 Six 15 ml subsamples of each water sample were then transferred using a pipette into sterile tubes containing 35 ml of ethanol to preserve the eDNA sample.

2.1.9 Samples were then removed from site and stored in a refrigerator before being sent to the laboratory for analysis.

Laboratory protocol

2.1.10 Natural Resources Wales (NRW) have produced a guidance sheet for eDNA analysis, *The use of environmental DNA test for Great crested newt licensing purposes* (Annex A) (NRW ND.). The guidance sheet refers to a Technical Guidance Note published by the Department for Environment Food and Rural Affairs (Defra), namely *WC1067 Analytical and Methodological Development for Improved Surveillance of The Great Crested Newt, Version 1.1* (Biggs *et al*., 2014). SureScreen Scientifics undertake laboratory analysis in accordance with the Defra Technical Guidance Sheet - WC1067 (Version 1.1) (as reported on sheet 2 of Annex B, under *Methodology*).

2.1.11 The protocol for laboratory analysis requires that laboratories undertaking eDNA analysis should be able to demonstrate adequate quality assurance standards which would typically comprise a documented quality management system. SureScreen Scientific are ISO 9001 accredited.

2.1.12 The laboratory methodologies followed those described by Biggs, et al. (2014) and included appropriate precautions to avoid laboratory contamination and minimise the risk of false positive and negative results. Details of the laboratory standards in place at SureScreen Scientifics are included in Annex C.

3 Results

3.1 Results of eDNA Laboratory Analysis

3.1.1 Results of the analysis of the water samples are summarised in Table 1 below. The laboratory result sheet provided by SureScreen Scientifics is attached at Annex B.

Table 1: Summary of eDNA analysis results

Waterbody	% of perimeter accessible	Flow	Notes	eDNA Result
Pond 1	80%	n/a	Shallow pond/pooled area of water on hard-standing, very small amount of aquatic/semi-aquatic vegetation and restricted to northern edge only, signs of some limited pollution, very minimal slope to profile, high likelihood of drying out during the summer months.	**Negative**
Pond 2	50%	n/a	Shallow pond/pooled area of water on hard-standing, very little aquatic/semi-aquatic vegetation (majority of waterbody has no vegetative cover), little to no slope to margins and profile, signs of moderate pollution, moderate likelihood of drying out during the summer months. Dense scrub along northern margin prevented access to the northern half.	**Negative**
Reen	75%	very slow	Heavily vegetated field ditch with steep banks, moderate amounts of submerged vegetation, no signs of pollution, low likelihood of drying out during summer months. Unable to collect samples from the eastern section due to steep banks and dense vegetation; however, remaining stretches accessible.	**Negative**

3.1.2 Results of the laboratory eDNA analysis reported no positive records of eDNA for great crested newts in any of the waterbodies surveyed.

4 Discussion

4.1 False Negatives

4.1.1 Three main factors have been found to lead to false negatives (Biggs, *et al.*, 2014):

- waterbodies with very low numbers of newts;
- waterbodies with wide shallow edges; and
- waterbodies where sampling was restricted to a small part of the waterbody.

4.1.2 In addition, Biggs *et al*. (2014) identify two main actions which may help to reduce false negatives:

- providing face to face training for surveyors; and
- using appropriate survey methods that ensure samples are collected from as much of the waterbody as possible, avoiding very shallow water (e.g. less than 5 cm deep) and very densely vegetated areas.

4.1.3 Therefore, in order to reduce the risk of false negatives, surveyors collecting the eDNA samples were adequately trained in sample collection methods and informed of the need to avoid shallow and densely vegetated water where practicable. Waterbodies across the site were typically limited in aquatic vegetation. Therefore, the likelihood of false negatives was reduced as far as possible.

4.2 Conclusions and Recommendations

4.2.1 The survey area is unlikely to sustain a population of great crested newts due to the limited amount of suitable habitat and isolated nature of the site, being surrounded by heavily built-up areas and the docks to the north, the River Usk to the south and east, and the River Ebbw to the west. Furthermore, ponds and drainage ditches are of low potential value to breeding newts due to the limited amount of aquatic/semi-aquatic vegetation and largely ephemeral nature - during the survey visit, all but the three waterbodies that were surveyed were dry.

4.2.2 Taking the above into account, along with the fact the survey area is isolated by the River Usk from other known great crested newt populations (Appendices 10.6 and 10.22 to the March 2016 M4CaN Environmental Statement), and considering that surveyors were fully trained in the method of collecting water samples, it is unlikely that results of the water analysis were false negatives, and it is concluded that great crested newts are absent from the survey area.

4.2.3 No further presence/absence surveys are considered necessary.

References

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R.A., Foster, J., Wilkinson, J., Arnett, A., Williams, P., Dunn, F. (2014) Analytical and methodological development for improved surveillance of the Great Crested Newt. *Defra Project WC1067. Freshwater Habitats Trust: Oxford*.

Natural Resources Wales (no date). The use of Environmental DNA Test for Great Crested Newt Licencing Purposes.

Figure 1: Survey Locations

Pond 1

Length of reen surveyed

Pond 2

Legend

- Land Parcels
- Species poor hedgerow
- Non- native treeline
- Surveyed waterbodies
- Broadleaved woodland
- Intertidal mud/ sand
- Saltmarsh habitats (NVC required)
- Drainage ditch
- Standing water (ephemeral)
- Reed bed
- Establishing grassland on made ground
- Amenity grassland
- Building
- Hardstanding
- Ephemeral/ short perennial
- Bare ground
- Scrub dense continuous
- Scattered scrub
- Tall ruderal
- Rubble mound (unvegetated)
- Rubble mound
- Target note

Llywodraeth Cymru
Welsh Government

Appendix ESS5 2.5

2017 Great Crested Newt eDNA Survey Locations

Figure: 1	Revision: 1
Date: August 2017	Status: AT ISSUE
Drawn: BM	Checked: JW

Scale: A3 @ 1:4,000

0 65 130 m

N

dwg ref: 8235-0706-09

Annexes

Annex A: The Use of Environmental DNA Testing for Great Crested Newt Licensing Purposes

Cyfoeth Naturiol Cymru
Natural Resources Wales

The use of environmental DNA test for Great crested newt licensing purposes

The Department for Environment Food & Rural Affairs (DEFRA) has recently published the results of an investigation into the use of environmental DNA (eDNA) to detect the presence of Great crested newt (GCN) in water bodies together with a technical advice note setting out the field and laboratory methodology DEFRA Science and Research Project WC1067.
http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&ProjectID=18650&FromSearch=Y&Publisher=1&SearchText=wc1067&SortString=ProjectCode&SortOrder=Asc&Paging=10#Description

On the basis of this study Natural Resources Wales (NRW) will now accept eDNA test results as evidence of presence or absence of GCN for licence

Key Findings of the report (this is not comprehensive so please read the 'final report' and 'technical advice note'):

- eDNA can have a better rate of GCN detection (99%) than a combination of conventional survey techniques ((95%);
- It will detect GCN presence or absence within a water body up to 7 – 21 days after newts utilising it;
- It requires 1 day time visit to each water body but the visit must be targeted when GCN are likely to be present in water bodies in the area (which may change on a yearly basis depending on local / regional conditions); and
- The study is based only on a single year and the 'final report' states samples were taken from late April to late June. (Note for 2014 NRW will accept samples taken between 15th April to 30th June – see below).

For GCN licence applications we anticipate this technique will be useful for developments/projects with a **long lead in time** where it can be used for early stage assessments and screening to determine the water bodies that require population size class assessment surveys, and for temporary and low impact cases which may require presence or absence surveys only. This is because it can take several weeks for results to be available from the analytical process.

When using this technique to support a licence application you should be aware that:

1. Use of eDNA is just another survey technique – it is not a mandatory requirement. We will accept this new technique to determine GCN presence or absence if samples are undertaken in strict accordance with the published technical advice note and they are collected by a suitably trained and experienced licensed GCN surveyor. (Note that a survey licence is not

required to take the water samples, but for licence applications we will require evidence and confirmation that experienced, licensed GCN surveyor/s collected the samples to support the proposals in the method statement).

2. Applicants wishing to submit eDNA test results as evidence of presence or absence of GCN, must declare that:

    a. They have strictly followed the technical advice note;

    b. Only licensed GCN surveyors (provide names and references) have taken the samples to support their licence application; and

    c. Present the field and laboratory results as part of their application, by including a separate WORD document with the application clearly setting out

        i. The referenced water bodies which were tested,

        ii. Dates that samples were taken, and

        iii. The results (presence or absence) in tabular form - which must also be reflected on the survey maps/figures submitted (see point 3).

3. Method statements must also include on the relevant survey figure/s water bodies sampled and surveyed, clearly indicating water body references and results (presence or absence).
4. During 2014 we are only accepting eDNA results from samples collected following the onset of suitable weather conditions for surveying GCN between the 15th April and 30 June.
5. This technique will not provide population size class assessments.
6. Should a population size class assessment be required for the proposed development/project then the applicant will require 6 survey visits using conventional survey methods, in accordance with current recommendations within the "*Great Crested Newt Mitigation Guidelines, 2001*".
7. Our *Information to be provided in a European Protected Species Licence application form* document will be adapted in due course, but untill then this advice on what we will typically expect if an applicant wishes to use this technique is to be followed. Should population size class estimates be required then the survey section of the method statement must be completed as usual.
8. Applicants must ensure they retain or have access to the records set out in the technical advice note, and used to support the licence application, for at least 12 months following the first licence return (date of which will be set out in any licence granted).
9. eDNA can also be used for post development monitoring surveys if presence or absence only surveying is required under licence.

The study undertaken did not include an analysis of potential suppliers or likely level of demand. Therefore should you wish to use this test you will need to make an informed **risk based judgement** about whether to use this test or conventional surveys to detect presence and absence of GCN. In particular applicants should be aware of:

- Factors affecting false negatives when collecting water samples – the need for training and recognising there will be difficult or less suitable sites for this technique.
- Factors causing false positives within handling and laboratory analysis – the results were obtained using a high specification laboratory. There is a risk that other laboratory layouts might generate different results. When commissioning laboratory analysis users should satisfy themselves that they can achieve a satisfactory level of performance.
- The study is based on a single years results and expert judgement should be applied to ensure samples are collected at the optimum time, when GCN are active, bearing in mind geographical location and conditions early in the year.
- Whether their project timetable allows sufficient time to undertake the required number of population size class assessment surveys (i.e. the conventional 6 surveys between March-June). If an eDNA test shows presence of GCN and as such a population assessment is required for the proposed development and impacts. This will require careful forward planning.
- The study looked at one type of eDNA test – quantitative Polymerase Chain Reaction (qPCR). We anticipate that variations in the technique from those studied and new techniques will emerge if industry finds this test useful. Industry will need to demonstrate equivalence for alternative tests.

For the immediate future we will therefore only accept eDNA evidence using the specific qPCR test set out in the technical advice note.

We will monitor how much uptake there is of this technique and seek feedback from industry on their experience.

Annex B: eDNA Survey Results

SureScreen Scientifics

Folio No: E1534
Report No: 1
Order No: 0307830
Client: RPS GROUP
Contact: Sean Flynn
Contact Details: sean.flynn@rpsgroup.com
Date: 19/07/2017

TECHNICAL REPORT

ANALYSIS OF ENVIRONMENTAL DNA IN POND WATER FOR THE DETECTION OF GREAT CRESTED NEWTS

Date sample received at Laboratory: 06/07/2017
Date Reported: 19/07/2017
Matters Affecting Results: None

RESULTS

Lab Sample No.	Site Name	O/S Reference	SIC	DC	IC	Result	Positive Replicates
32011	M4CAN 06	-	Pass	Pass	Pass	Negative	0
32195	Caldicot A	-	Pass	Pass	Pass	Negative	0
32197	Duffryn 5	-	Pass	Pass	Pass	Negative	0
32198	Caldicot B	-	Pass	Pass	Pass	Negative	0
32200	ABP Reen 1	-	Pass	Pass	Pass	Negative	0
32004	M4 Can D4	-	Pass	Pass	Pass	Negative	0
32207	Dufryn 3	-	Pass	Pass	Pass	Negative	0
32208	Pond 2 ABP	-	Pass	Pass	Pass	Negative	0
32211	Duffryn 2	-	Pass	Pass	Pass	Negative	0
32216	ABP Pond 1	-	Pass	Pass	Pass	Negative	0
32218	Duffryn 1		Pass	Pass	Pass	Negative	0

SUMMARY

When Great Crested Newts (GCN); Triturus cristatus inhabit a pond, they deposit traces of their DNA in the water as evidence of their presence. By sampling the water, we can analyse these small environmental DNA (eDNA) traces to confirm GCN habitation, or establish GCN absence.

The water samples detailed below were submitted for eDNA analysis to the protocol stated in DEFRA WC1067 (Latest Amendments). Details on the sample submission form were used as the unique sample identity.

RESULTS INTERPRETATION

Lab Sample No.- When a kit is made it is given a unique sample number. When the pond samples have been taken and the kit has been received back in to the laboratory, this sample number is tracked throughout the laboratory.

Site Name- Information on the pond.

O/S Reference – Location/co-ordinates of pond.

SIC- Sample Integrity Check. Refers to quality of packaging, absence of tube leakage, suitability of sample (not too much mud or weed etc.) and absence of any factors that could potentially lead to results errors. Inspection upon receipt of sample at the laboratory. To check if the Sample is of adequate integrity when received. Pass or Fail.

DC- Degradation Check. Analysis of the spiked DNA marker to see if there has been degradation of the kit since made in the laboratory to sampling to analysis. Pass or Fail.

IC- Inhibition Check- PCR inhibitors can cause false results. Inhibitors are analysed to check the quality of the result. Every effort is made to clean the sample pre-analysis however some inhibitors cannot be extracted. An unacceptable inhibition check will cause an indeterminate sample and must be sampled again.

Result- NEGATIVE means that GCN eDNA was not detected or is below the threshold detection level and the test result should be considered as no evidence of GCN presence. POSITIVE means that GCN eDNA was found at or above the threshold level and the presence of GCN at this location at the time of sampling or in the recent past is confirmed. Positive or Negative.

Positive Replicates- To generate the results all of the tubes from each pond are combined to produce one eDNA extract. Then twelve separate analyses are undertaken. If one or more of these analyses are positive the pond is declared positive for the presence of GCN. It may be assumed that small fractions of positive analyses suggest low level presence but this cannot currently be used for population studies. In accordance with Natural England protocol, even a score of 1/12 is declared positive.

METHODOLOGY

The laboratory testing adheres to strict guidelines laid down in WC1067 Analytical and Methodological Development for Improved Surveillance of The Great Crested Newt, Version 1.1

The analysis is conducted in two phases. The sample first goes through an extraction process where all six tubes are pooled together to acquire as much eDNA as possible. The pooled sample is then tested via real time PCR (also called q-PCR). This

process amplifies select part of DNA allowing it to be detected and measured in ‘real time’ as the analytical process develops. qPCR combines PCR amplification and detection into a single step. This eliminates the need to detect products using gel electrophoresis. With qPCR, fluorescent dyes specific to the target sequence are used to label PCR products during thermal cycling. The accumulation of fluorescent signals during the exponential phase of the reaction is measured for fast and objective data analysis. The point at which amplification begins (the Ct value) is an indicator of the quality of the sample. True positive controls, negatives and blanks as well as spiked synthetic DNA are included in every analysis and these have to be correct before any result is declared so they act as additional quality control measures.

The primers used in this process are specific to a part of mitochondrial DNA only found in GCN ensuring no DNA from other species present in the water is amplified. The unique sequence appropriate for GCN analysis is quoted in DEFRA WC 1067 and means there should be no detection of closely related species. We have tested our system exhaustively to ensure this is the case in our laboratory. We can offer eDNA analysis for most other species including other newts.

Analysis of eDNA requires scrupulous attention to detail to prevent risk of contamination. Kits are manufactured by SureScreen Scientifics to strict quality procedures in a separate building and with separate staff, adopting best practice from WC1067 and WC1067 Appendix 5. Kits contain a ‘spiked’ DNA marker used as a quality control tracer (SureScreen patent pending) to ensure any DNA contained in the sampled water has not deteriorated in transit. Stages of the DNA analysis are also conducted in different buildings at our premises for added

SureScreen Scientifics Ltd also participate in Natural England’s proficiency testing scheme and we also carry out inter-laboratory checks on accuracy of results as part of our quality procedures.

Reported by: Derry Hickman **Approved by:** Sam Humphrey

End Of Report

Annex C: Laboratory Standards and Procedures

SureScreen Scientifics

Technical Bulletin
Issue 5

e-DNA FOR GREAT CRESTED NEWT 'EVIDENCE OF PRESENCE'

The Great Crested Newt, *Triturus cristatus*, or GCN, is a newt in the family Salamandridae whose numbers are declining in most of Europe due to loss of habitat, fragmentation, deteriorating pond systems or poor water quality.

As with other protected species, the GCN is a valuable marker of the local ecological balance. It is a protected species under Sch.5 of the Wildlife and Countryside Act 1981, with equivalent legislation in Northern Ireland. It is also a European Protected Species and it has additional protection in the UK under Regulation 39 of the Conservation (Natural Habitats etc.) Regulations 1994. As a result, planning laws require an evaluation of any site where GCN presence is possible.

In 2014 the use of eDNA to record species occupancy in ponds was approved in some EU countries for a range of species that included GCN.

eDNA is DNA that is collected from the environment in which an organism lives, rather than directly from the organism itself. In aquatic environments animals including amphibians and fish shed cellular material into the water via their saliva, urine, faeces, skin cells etc. This DNA may persist for several weeks, and can be collected through a water sample, and analysed to determine if target species of interest have been present. Furthermore, using this technique it is possible to detect multiple species from the same water sample.

Thomsen et al (1) showed that GCN eDNA in water degrades in the pond in about 20 days, so a positive result shows the species has been present recently.

eDNA BENEFITS

- Evidence of presence valid for previous 20 days
- Sample any time of day
- More accurate than surveys
- Less Intrusive and safer
- Perfect for 'murky water'
- Accurate for 'evidence of presence'
- Negatives give good guidance
- One sample, multiple species
- qPCR technique may determine population densities
- Future data possibilities.

The eDNA technique has a number of potential advantages, summarised in the Table. The GCN is an example of a fairly cryptic pond species, with a relatively low detection rate using traditional methods of sampling. That's why several negative repeat samples of the same site are needed to provide a reasonably high confidence that the species is truly absent from the site. With eDNA a negative result will be a much stronger indication of true absence, and any individual GCN that is in the pond has a higher likelihood of being detected, even in conditions that are not conducive to traditional sampling (e.g. murky waters).

WHEN CAN WE TEST?

Theoretically, tests can be done at any time, and a positive detection of eDNA is a sure sign of GCN occupancy. However, because GCN activity is mostly land-based and ponds are used for displaying and breeding, negative results will only mean the pond is not a GCN habitat within that breeding period.

Currently, for presence/absence surveys, eDNA can only be sampled between 15 April and 30 June. Although samples taken outside this period can show presence (say, if larval newts are in a pond) such samples cannot be used to prove absence. Currently, these dates are determined in advance and do not take account of weather and regional variations. It seems likely then, that as we develop the methodology further, regional variations in seasons could be addressed. In fact ecologists are well aware of activity stirring after Winter and might be able to make their own decisions within a wider window using the more exact methodology of eDNA.

Although this is an exciting new technological advance for environmental consultancy, at the moment eDNA will not provide an accurate and reliable idea of population size (i.e. numbers of newts present) so, where licencing requires it conventional survey techniques are likely to be more appropriate. However our analytical methods with quantitative PCR certainly opens up this possibility in future. This is something we're going to be researching in conjunction with Dr Michael Sweet, from the College of Life and Natural Sciences, at the University of Derby.

Although a licence is not required to take water samples, care is needed, and Natural England has advised that eDNA water samples would need to be collected by a licenced newt surveyor if survey results are to be provided in support of an European Protected Species (EPS) licence application (presumably to demonstrate proof of absence, where relevant).

You're breaking the law if you:

- capture, kill, disturb or injure great crested newts (deliberately or by carelessness).
- damage or destroy a breeding or resting place (even accidentally).
- obstruct access to their resting or sheltering places (on purpose or by not taking enough care).
- possess, sell, control or transport live or dead newts, or parts of them.
- take great crested newt eggs.
- If you're found guilty of an offence you could be sent to prison for up to 6 months and be fined £5,000 for each offence.

HOW LONG DOES IT TAKE?

Although the sample is preserved in alcohol, eDNA deteriorates with time, so samples are best kept chilled in a cool box, placed in the fridge overnight, and couriered to us with an ice pack. Our sample kit is guaranteed DNA-free and simplifies everything. We extract the DNA from the sample as soon as we receive it and freeze the extract until we test it. Although the testing process takes a day, many laboratories wait until they have enough samples to make the cost economic, so results can sometimes take months. That's a problem because you then have no option but to continue with surveys while you wait for the answer. We appreciate time is critical so we analyse samples within a week, and even offer a premium service that gives you result within three days of receipt of sample. This service is unique and we can only offer this fast turnround because we routinely carry out other forms of DNA analysis in our laboratory.

HOW ACCURATE IS IT?

The process of detecting eDNA provides positive evidence of presence, so this is a great tool in your portfolio to prove GCN are in that pond without the uncertainty of surveys. Protocols were developed with a great deal of research, (2) which showed an accuracy of 99.3% compared to surveys which were only 75% accurate.

eDNA appears to be a highly effective method to determine whether Great Crested Newts are present or absent during the breeding season.

At the present time, a negative result can't be used as conclusive evidence that GCN are NOT present. The reasons for this centre around the uncertainty of complete sampling, especially where access round the whole pond is limited. In those cases, routine surveys or more eDNA samples taken periodically are necessary. Even so, this is a valuable tool in the ecologist's portfolio that is bound to become more accepted and more prevalent with time. Our research is helping that process.

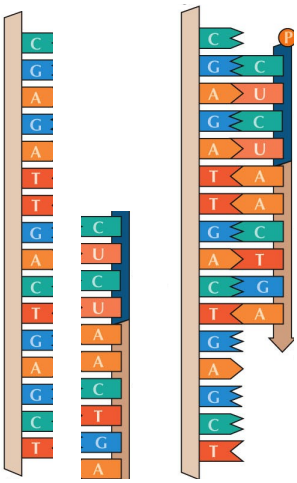
eDNA - HOW IS IT DONE?

All species have unique DNA, constructed from amino acid 'rungs' on a complex carbon ladder system. That sequence of billions of amino acids is what makes every species unique. Subtle variations or polymorphisms in that sequence are what make you and I different. We all leave traces of our DNA around us, just like GCN presence in a pond leaves DNA traces in the water.

Once the unique sequence is known for a species, we can manufacture de-novo sections of that DNA sequence known as a *primer* that will bond to the RNA and initiate a replication sequence. We use a Polymerisation Chain Reaction (PCR) to separate the strands of DNA into its two ladder strings of RNA, allowing primer bonding, then we amplify the DNA in the chain reaction and

analyse it. If GCN DNA is present, the primer reacts with it and produces readable levels of newt DNA. If none is present, the primer has nothing to bond with so no amplification occurs. The rate of amplification that is detected depends on the amount that was present, so theoretically an estimate could be made of the population density, but this depends on accurate sampling of the pond. Early work used traditional PCR which was read at the 'end point' of the process for presence and absence. We only use the protocol approved by Natural England which requires real-time quantitative PCR in which the sample is analysed continuously by the instrument, known as qPCR.

This drawing shows how a primer works. On the left is a tiny portion of a DNA sequence unique to GCN. In the centre is a 'de-novo' or 'assembled' primer made specifically to pair with that unique portion of DNA. The right hand image shows that primer paired with the unique newt DNA, which once amplified and read, provides absolute evidence of that unique eDNA in the sample. Only the GCN has that unique code so there can be no false positives provided sampling avoided cross contamination.



We're used to suiting up for a crime scene, to avoid spreading DNA around. In the case of eDNA, the technology is well proven, and it is the care of the ecologist that provides the best accuracy. This is achieved by avoiding passing eDNA from one pond to another, and ground eDNA into water, or stirring up old DNA in mud or sediment. We've simplified Natural England's advice in the Table opposite. Follow these guidelines in conjunction with our forensic skills and advice, you'll serve your clients better, get results quicker, and provide a state of the art service that will be hard to match in accuracy, speed of turn round and value for money.

Risks	Prevention
False Positives	
Cross contamination	Take care to avoid contact from other ponds via boots, tools etc. Use only Scientifics sampling kits which are eDNA-free. If you use a pole, do not allow it to get wet and contaminate other sites.
Inflows of eDNA from other ponds etc. Unlikely but theoretically possible.	Most GCN ponds have no inflows. Record the presence/absence of inflows in the survey.
Aquatic animals e.g. herons, water voles; transferring eDNA between sites.	Risk is small. Recording habitat details will help us refine the eDNA process and offer more advice. We'll freeze samples for future statistical research too so you'll be helping to improve the protocol.
False Negatives	
Low numbers of newts may result in low eDNA counts.	Follow good field protocol, refrigerate samples overnight and use our courier service for prompt delivery to avoid deterioration. We use qPCR to detect low levels of eDNA, optimizing accuracy in low levels.
Samples from deep ponds may not have eDNA in them even if the pond is occupied.	Gently stir the water as eDNA is believed to sink. Avoid water less than 5-10 cm deep. Tape the ladle to a long pole for deep water. Do not stir sediment which may contain historical eDNA.
eDNA is less likely in densely packed mats of vegetation.	Sample water in areas where vegetation is suitable for egg-laying and open water areas suitable for displaying.
eDNA is less likely to be detected if the whole pond perimeter is not sampled.	Try to access 20 sampling positions, as where 80-90% of pond margins were accessed, testing achieved 99.3% detection rates.

WHO ARE WE?

You may not have heard of SureScreen Scientifics before, because we're usually the backroom boffins behind some of the world's leading brands. In fact many of our clients have their own well equipped laboratories but still utilise our special skills.

Our diagnostics arm manufactures many of today's modern medical tests for issues such as pregnancy, cancer and infectious diseases. Our Life Sciences Lab analyses neurochemistry associated with dementia, schizophrenia and depression, and our forensics lab undertakes examinations of crime scenes & engineering disasters.

Our DNA suite analyses biological materials. We currently have two DNA research projects. The first involves a UK team dedicated to developing a rapid genetic sequencer for tumour tissue that should provide rapid results on the tumour type and its response to chemotherapy in a fraction of the time taken for our standard sequencer and at a fraction of the cost. Our second project, RESPOC undertakes genetic analysis of respiratory diseases such as pertussis (whooping cough) to detect the problem in infants earlier than ever before, because early diagnosis is the key to keeping potentially deadly respiratory diseases at bay especially in the young.

ANY QUESTIONS?

Why are sampling points spread around the pond?
eDNA level depends where GCN have been. Sampling many areas increases the likelihood of collecting their DNA successfully.

Why is the water column mixed before sampling?
DNA tends to sink so there will be more nearer the bottom. However, do not collect sediment as old DNA might be present, causing a greater risk of a false positive.

Why is such a large volume of water collected?
Natural England recommends a larger volume of water than previous methods (1) as it improves accuracy. Mix this quantity and fill the six small tubes in the kit from it to send to us for analysis.

What if duckweed, algae or zooplankton are in my sample?
Small amounts don't matter. We only detect GCN eDNA.

What if I spill the preservative - or the sample tube itself
If you spill some preservative from one of the tubes, just add proportionally less water from your pond sample. We combine all the samples, so it's not disastrous if some sample is lost. The preservative is alcohol, which isn't hazardous.

Will samples degrade in the post?
Keeping the samples refrigerated slows DNA degradation. Add an ice pack if you can, but the rate of decay while posting at ambient temperatures will not degrade the sample completely.

What evidence is there to support the use of this technique?
Defra project WC1067 revealed GCN eDNA 99.3% of the time in ponds where they were known to occur. When used by volunteer surveyors, eDNA detected Great Crested Newts at 91% of ponds where they were known to be present. No false positives were recorded from sites either outside or within the known range of the newt. It is the method, not the laboratory, which is approved, but we are working with the University of Derby to develop a quality control auditing scheme for participating laboratories to keep standards high and develop the technology further.

REFERENCES

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2. http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=0&ProjectID=18650 (refer to downloads)

We're well known for delivering advice that is simple to understand and easy to apply. Why not check out our other bulletins, or browse our extensive library of failures on www.surescreenscientifics.com.

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JUNE e-DNA UPDATE / OTHER SERVICES / R&D RELIEF

The Great Crested Newt, *Triturus cristatus*, or GCN, is an ideal subject for eDNA surveying methods. Rather than surveying the presence or absence of the organism itself, environmental DNA or eDNA can be detected from the organism's cellular material via their saliva, urine, faeces, skin cells etc. This eDNA may persist for several weeks. Thomsen showed in 2012 that GCN eDNA in water degrades in the pond in about 20 days, so a positive result shows the species has been present recently.

In our earlier bulletins we explained the process of sample collection and some of the theory behind eDNA. Here, we touch on some of the quality issues behind the lab testing and 'behind the scenes' work that we undertake to ensure you receive accurate result in a timely manner. We're hands-on forensic scientists, and we know how difficult it can be to sample and how demanding clients can be.

LAB TESTING

The laboratory testing is conducted in three phases, and each phase is zoned in the laboratory to prevent airborne contamination. DNA analysis is extremely sensitive so to avoid any risk of contamination the area is confined to authorized staff and samples are segregated. Each kit is booked into the laboratory system on receipt so the analysts have no knowledge of the client or location of the sample. (Our Laboratory also analyses urine and blood from famous sportspeople but only the Laboratory Director knows their identity).

Samples remain refrigerated throughout their journey in the Laboratory, and even the ultracentrifuges we use are refrigerated to 4 degrees Celsius. The kit first goes through an extraction process where all 6 tubes are centrifuged and the eDNA is extracted and pooled together to acquire as much as possible from the pond. The pooled sample is then reacted with an enzyme and fluorescence labelling system which finds, reacts with and labels the GCN eDNA. This process is lengthy which is why even our 'fast track' system takes a few days.

eDNA BENEFITS

- **Evidence of presence valid for previous 20 days**
- **Sample any time of day**
- **More accurate than surveys**
- **Less Intrusive and safer**
- **Perfect for 'murky water'**
- **Accurate for 'evidence of presence'**
- **Negatives give good guidance**
- **One sample makes it easy**
- **qPCR technique may determine population densities**
- **Future data possibilities.**

Now comes the clever bit. An aliquot from this reacted sample is tested via real time PCR (or q-PCR). This process not only amplifies select part of DNA allowing it to be detected and measured, it continuously tracks the increase in target DNA and plots the result against known controls and any other calibration DNA we have introduced for quality control purposes. Each kit we receive is sampled and replicated in the instrument 12 times to ensure results are accurate. All samples are randomly distributed in the array of samples which include negative and positive controls and calibrators. These non-sample controls and calibrators are necessary whether we have one sample or six samples to run, making it very expensive to test a single sample. That's probably why other labs have a slow turn-round of results, they probably wait for more samples to arrive before conducting a run, but while we lose money on small batches we believe it's the speed of service that is critical to the success of the eDNA service.

The q-PCR works in cycles, which are made up of different stages. The DNA is heated to break the bonds and make 2 separate complementary single strands of DNA. Primers that are specific to a section of the target DNA are then attached at a lower temperature. Once attached these primers act as a starting point for the polymerase enzyme, this completes the missing DNA creating two exact copies of the target sequence. This cycle is repeated so that any samples that have the target DNA sequence are amplified exponentially. At the end of every cycle the amount of DNA is measured giving a real time picture of the amplification process. The primers used in this process are specific to a part of mitochondrial DNA only found in GCN ensuring no other DNA is amplified. If one or more of the 12 replicates tests positive the sample is declared positive. The sample is only declared negative if no replicates show amplification.

WHEN CAN WE TEST?

Currently, for GCN presence/absence surveys, Natural England dictate that eDNA can only be sampled between 15 April and 30 June. However, samples taken outside this period can be used to show presence (say, if larval newts are in a pond) but negative results cannot be used to prove absence. Even so, a positive result can be helpful in large planning exercises where the developer might consider an alternative scheme to avoid inhabited ponds.

WHAT OTHER SERVICES ARE ON OFFER?

We have a fully equipped forensic laboratory that serves many industries. We handle water quality, contamination, planning issues, and construction materials as well as component failures and analysis. We're used by many well known companies as their centre of excellence. Here are a few examples of how we can serve you better and impress your clients:

- *Microscopy, including sharp, high definition images of fecal matter, frass, debris, contamination etc up to x10,000 magnification.*
- *Analysis of debris, particles, contaminants etc.*
- *'Source of origin' or 'who pollutes?' on unknown samples.*
- *Drone surveys with report-ready stills or videos.*
- *Bat species eDNA for bat droppings including photomicrographs*
- *Crayfish, Water Vole, Badger, Otter eDNA; others in test phase*
- *Research partnerships using Scientifics as your lab resource*

RESEARCH AND DEVELOPMENT TAX REBATE

Did you know that ecological advice for clients may sometimes qualify for the R&D tax rebate, making your fees very tax-efficient?

For Small and Medium Enterprises, the Research and Development Expenditure Credit (RDEC) Scheme runs alongside the Large Company enhanced-deduction scheme which it will replace in April 2016. Tax relief is given on eligible projects as an 11% taxable credit on the amount of qualifying R&D expenditure. If your company is small or medium-sized, you may be able to claim R&D relief under the SME Scheme for one project and the Large Company Scheme for another.

For larger companies, the tax relief on allowable R&D costs is 130% - that is, for each £100 spent, your client could have the income on which Corporation Tax is paid reduced by an additional £30 on top of the £100 spent. If instead there's an allowable trading loss for the period, this can be increased by 30% of the qualifying R&D costs - £30 for each £100 spent. This loss can be carried forwards or back in the normal way that deferred losses work.

This means that where surveys are done as part of research or development, especially when supported by eDNA results, your fees will be rebated by the R&D tax allowance. Government introduced this rebate to oil the wheels of commerce, industry and improve the environment so everyone wins.

For more info call or email tom.wood@surescreen.com.
Check out our other bulletins, or browse our extensive range of information on www.surescreen.com
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