Amenity Tree Care Ltd.

Land at Station Road, Reddish, Stockport, Greater Manchester - Great Crested Newt Survey

(November 2025)

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1.0 EXECUTIVE SUMMARY

1.1 Amenity Tree Care Ltd. was commissioned to undertake a great crested newt (GCN) aquatic survey and terrestrial habitat appraisal, assess the predicted development effect and formulate appropriate and proportionate GCN/amphibian mitigation and habitat enhancement measures in connection with the development/re-development of land at Station Road, Reddish, Stockport, Greater Manchester. Proposed redevelopment of the site includes the erection of new residential properties, restoration of one of the water bodies, creation of new vehicular, pedestrian and cycle links throughout the site. Site-wide landscaping including lakeside paths, seating areas and fishing huts.

1.2 The proposed development site extends to a total of approximately 4.75 hectares and is bounded to the north by Station Road and a disused railway line, (now a footpath), to the east by housing, to the south by playing fields, football pitches and residential properties and some wooded areas and to the west by light industrial properties.

1.3 At the time of the survey terrestrial habitats to be directly impacted by the proposed development comprised developed land/sealed surface, neutral grassland, non-native and ornamental hedgerow, scrub (bramble and mixed), sparsely vegetated land and woodland. However, the proposed development will also impact the aquatic reedbed habitat in the Eastern parcel of land (considered an aquatic habitat for these purposes).

1.4 Information relating to the presence of GCN and other amphibians within the local area (defined by Natural England as within 10 kilometres) was sourced from Magic.gov, the presence of GCN was recorded in 2015 approximately 1km to the NE of the proposed development site.

1.5 All water bodies identified on OS mapping and aerial imaging of the local area as potentially extant within 500 metres of the area of works were considered as part of the Amenity survey effort.

1.6 The review of OS and aerial mapping/imaging and previous reports for the site and walk-over inspections of the proposed development site and neighbouring land by an Amenity ecologist on 16th June 2025 established that 2 water-bodies

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(Waterbodies 1 & 2) and occasional drainage ditches were extant on or within 250 metres of the proposed development site boundary.

1.7 The review of OS and aerial mapping/imaging and previous reports for the site and the walk-over inspection established that only one further waterbody (Water-body A) and other drainage ditches were likely also extant at distances of 250 metres up to 500 metres from the proposed development site boundary. This waterbody was not considered relevant to the appraisal/survey, due to its considerable distance/isolated location from the proposed development site. In addition, Magic Map stated that GCN Survey had been undertaken on this pond in 2018 when it was found not to support GCN.

1.8 Based on the terrestrial range of individual GCN (generally <250 metres, occasionally >500 metres, rarely >1 kilometre from their breeding site) and given the availability of potential dispersal habitat it was considered reasonable to conclude that only Waterbodies 1 & 2 were relevant to the appraisal/survey.

1.9 Other waterbodies, such as drainage ditches and/or brooks, were excluded from the GCN Survey due to their unsuitability to support GCN breeding.

1.10 The methods employed at Waterbodies 1 & 2 included netting, egg-search, terrestrial refugia search and GCN eDNA water sampling and analysis for GCN presence/absence, as appropriate to the timing of the visit and the conditions encountered.

1.11 The HSI score calculated for Waterbody 1 indicated ‘below average’ suitability for GCN (0.5). It was considered possible that this water body could support some limited amphibian breeding.

1.12 The HSI score calculated for Waterbody 2 indicated ‘good’ suitability for GCN (0.74). It was considered possible that this water body could support amphibian breeding.

1.13 Visual observations undertaken during the site walk-over inspection visit did not result in the discovery of any evidence of GCN/other amphibians, their eggs and/or larvae being observed at Waterbodies 1 & 2.

1.14 A search of the limited terrestrial refugia at the proposed development site during the walk-over inspection visit did not result in the discovery of any sheltering GCN or other amphibians.

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1.15 At the time of the survey terrestrial habitat to be directly impacted by the works was assessed to offer GCN and/or other amphibians with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Very Good'.

1.16 The survey robustly established the absence of GCN at Waterbodies 1 & 2 during the 2025 survey season.

1.17 Consequently, has been concluded that GCN were unlikely to be present within the area/s to be directly affected by the works.

1.18 It is possible that a potential amphibian breeding site or aquatic habitat will be impacted, as a consequence of the proposed development, most likely Waterbody 2. However, Waterbody 2 has been considered to dry sometimes, limiting its usefulness to breeding amphibians.

1.19 The proposed works are likely to result in some; partial destruction, modified management/resurfacing, etc., temporary disturbance/destruction and reinstatement of immediate (<50 metres), intermediate (>50 metres to <250 metres) and distant (>250 metres) terrestrial habitat associated with Water-bodies 1 & 2 and/or other ponds/water bodies in the local area (defined by Natural England as within 10 kilometres).

1.20 The proposed works are unlikely to increase the historic and existing level of habitat fragmentation at the site level and will not isolate any GCN population/s or adversely impact on their ability to migrate/disperse and reach their breeding sites.

1.21 However, the development proposals for the site are likely to increase (from current and historic levels) the risk of post-development human interference impacts on any GCN population/s and/or aquatic and terrestrial habitats associated with the local area.

1.22 The predicted development effect and scale of impact have been assessed, with reference to the English Nature Great Crested Newt Mitigation Guidelines 2001 and, in the absence of appropriate mitigation measures, a 'potentially medium' negative development impact was predicted for any GCN/amphibian populations potentially associated with the local area.

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1.23 Based on the survey findings it has been concluded that GCN are unlikely to be present at the proposed development site. Consequently, the proposed development is considered unlikely to result in a breach of current wildlife legislation and/or Natural England guidance relating to the species and its shelter habitat.

1.24 Given that the proposed development will not have a significant or adverse impact on the favourable conservation status of any known GCN populations it has been concluded that GCN are unlikely to pose a constraint to the proposed development.

1.25 However, it is considered that the development could potentially have an adverse impact on any amphibians not encountered during the survey should they inadvertently stray onto the development site following the commencement of development and take cover under/within shelter habitat incidentally created as a consequence of the proposed development works, i.e. areas of disturbed ground, excavations, spoil mounds and/or stored materials.

1.26 Whilst the more common amphibians do not have the same legal protection as GCN, it is considered appropriate to recommend a mitigation strategy involving the implementation of amphibian Reasonable Avoidance Measures (RAM) in order to reduce potential development impacts on these species and to enhance the site for these species post-development, (in line with NPPF).

1.27 The predicted development effect and scale of impact have been assessed, and appropriate reasonable avoidance measures and enhancement measures have been formulated. It should therefore be possible for the Local Planning Authority to make an informed decision as to whether it is possible to discharge their responsibilities under current planning guidance, in respect of GCN/amphibians and biodiversity, by the implementation of planning condition/s when determining the planning application submitted in respect of the works.

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2.0 INTRODUCTION

2.1 Amenity Tree Care Ltd. was commissioned to undertake a great crested newt (GCN) aquatic survey and terrestrial habitat appraisal, assess the predicted development effect and formulate appropriate and proportionate GCN/amphibian mitigation and habitat enhancement measures in connection with the development/re-development of land at Station Road, Reddish, Stockport, Greater Manchester (refer to Appendix A: Site Location Plan (OS), Grid Ref: SJ 8874 3945). Proposed redevelopment of the site includes the erection of new residential properties, restoration of one of the water bodies, creation of new vehicular, pedestrian and cycle links throughout the site. Site-wide landscaping including lakeside paths, seating areas and fishing huts.

2.2 The Amenity 2025 survey was led by Dawn Goodfellow, BSc (Hons) MCIEEM (Senior Ecologist), accompanied by Mike Bazley. Dawn holds a Natural England GCN survey licence (Class Licence WML-CL08, Registration No.: 2015-16636-CLS-CLS) and is fully conversant with the English Nature Great Crested Newt Mitigation Guidelines 2001 and current legislation relating to the species. Dawn has extensive experience gained as the Appointed Ecologist on GCN licensed and non-licensed mitigation and GCN/protected species projects since 2001 and has undertaken training in collecting samples of water for GCN eDNA analysis (refer to Appendix A: Senior Ecologist's Experience and Appendix B: GCN and Protected Species Legislation).

2.3 The objective of the survey was to establish:

- The extent, status and relevance of aquatic and terrestrial habitats located at and within 500 metres of the proposed development site/area/s of works.
- GCN/amphibian presence/likely absence and population size class at the site level and within the local area.
- The predicted development effect and scale of impact on GCN/other amphibians.
- The likely impact of development on the favourable conservation status of any GCN/amphibian population/s associated with the local area.

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- Potential legal and licensing implications in respect of current wildlife legislation relating to GCN/other amphibians.
- Appropriate mitigation and habitat enhancement measures to address the predicted development effect and level of impact on GCN/other amphibians.

2.4 This report should enable the Local Planning Authority to make an informed decision as to whether or not it is possible to discharge their responsibilities under current planning guidance, in respect of GCN and biodiversity, by the implementation of planning condition/s when determining the planning application submitted in respect of the works (refer to Appendix C: Natural England Local Planning Authority GCN Standing Advice Guidance - Flowchart).

3.0 SITE DESCRIPTION

3.1 The proposed development site extends to a total of approximately 4.75 hectares and is bounded to the north by Station Road and a disused railway line, (now a footpath), to the east by housing, to the south by playing fields, football pitches and residential properties and some wooded areas and to the west by light industrial properties (refer to Appendix D: Site Location Plan (OS) & Appendix E: Photographic Plates).

3.2 At the time of the survey terrestrial habitats to be directly impacted by the proposed development comprised developed land/sealed surface, neutral grassland, non-native and ornamental hedgerow, scrub (bramble and mixed), sparsely vegetated land and woodland. However, the proposed development will also impact the aquatic reedbed habitat in the Eastern parcel of land (considered an aquatic habitat for these purposes).

3.3 The terrestrial habitat types at the area/s of works, their extent and suitability for GCN/amphibians are detailed in Table 1 below:

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Table 1: Site Habitat Type, Extent & GCN/Suitability for Amphibians

Habitat type	Approximate Area (Ha)/length	Suitability for GCN/Amphibians
Developed land/sealed surface	0.009	'Very Poor'
Neutral grassland	0.175	'Above Average'
Non-native and ornamental hedgerow	0.023	'Average'
Scrub (bramble/mixed)	0.890	'Good'
Sparsely vegetated land	0.185	'Below Average'
Woodland	1.729	'Very Good'

3.4 At the time of the site walk-over inspection visit terrestrial habitat to be directly impacted by the proposed development was assessed to offer GCN with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Very Good'.

3.5 The land habitat suitability categorisations used by Amenity are based on the Land Habitat Suitability Index (Land HSI) developed by Dawn Goodfellow BSc (Hons) MCIEEM (Senior Ecologist). The Land HSI is necessarily a broad interpretative tool that can only provide general guidance when categorising the suitability of terrestrial habitat for GCN/amphibians. The Land HSI relies implicitly on the experience and professionalism of the ecologist using the Index for consistency and accuracy. The Land HSI has been developed to help standardise GCN/amphibian terrestrial habitat assessments/categorisations and provide more accurate and consistent data to help inform the decision-making process relating to GCN/amphibian mitigation projects (refer to Appendix F: Land HSI).

3.6 All water bodies identified on OS mapping and aerial imaging of the local area as potentially extant within 500 metres of the area/s of works were considered as part of the Amenity survey effort.

3.7 The review of OS and aerial mapping/imaging and previous reports for the site and walk-over inspections of the proposed development site and neighbouring land by an Amenity ecologist on 16th June 2025 established that 2 water-bodies (Waterbodies 1 & 2) and occasional drainage ditches were extant on or within 250 metres of the proposed development site boundary.

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3.8 The review of OS and aerial mapping/imaging and previous reports for the site and the walk-over inspection established that only one further waterbody (Water-body A) and other drainage ditches were likely also extant at distances of 250 metres up to 500 metres from the proposed development site boundary. This waterbody was not considered relevant to the appraisal/survey, due to its considerable distance/isolated location from the proposed development site. In addition, Magic Map stated that GCN Survey had been undertaken on this pond in 2018 when it was found not to support GCN.

3.9 Based on the terrestrial range of individual GCN (generally <250 metres, occasionally >500 metres, rarely >1 kilometre from their breeding site) and given the availability of potential dispersal habitat it was considered reasonable to conclude that only Waterbodies 1 & 2 were relevant to the appraisal/survey.

3.10 Other waterbodies, such as drainage ditches and brooks, were excluded from the GCN Survey due to their unsuitability to support GCN breeding.

3.11 A brief description of the aquatic habitats at Waterbodies 1 & 2 (included in the GCN Survey) has been provided for clarity (refer to Appendix E: Photographic Plates).

Waterbody 1 (Grid Reference: SJ 88735 94620):

Approximate area 1.06ha, current water depth approx. >2m, this waterbody was located between residential, light industrial and amenity areas mainly surrounded by footpaths, scrub and woodland. It was less than 60% shaded around the margins. It is unlikely that this waterbody will be lost to the development. The waterbody supported a limited number and diversity of aquatic plants and water quality was considered to be poor. Waterfowl were present although impacts were considered to be minor. This waterbody was considered to never dry and due to the presence of local anglers and stations for angling around the margin's major impacts from the presence of fish were considered definite. It was considered possible that this water body could support amphibian breeding (likely common toad and potentially common frog and smooth newt), although no signs of amphibian presence were observed during the site visit.

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Waterbody 2 (Grid Reference: SJ 89039 94711):

Approximate area 0.58ha, current water depth approx. 0.4m, this area was located within the Eastern parcel of the proposed development site surrounded by woodland with amenity areas to the south of it. It is considered likely that this waterbody would be lost to the development. The waterbody supported a limited number and diversity of aquatic plants, mainly common reed. Water quality was considered to be poor due to regular drying. Impacts from waterfowl were considered to be minor and the presence of fish was thought possible considering the proximity of this waterbody to several residential areas. It was considered possible, though unlikely, that this water body could support successful GCN breeding (due to likely drying sometimes) but it could be possible in wetter years. It is also possible tat it could support other amphibian species such as smooth newt, common toad and common frog although no signs of amphibian presence were observed during the site visit.

3.12 During the site walk-over inspection visit and assessment of aerial mapping, it was noted that several, private, residential gardens were located within 500 metres of the area/s of works. It was considered possible that some of these gardens may also contain ponds/ornamental water features potentially used by GCN/amphibians. However, given the extensive experience of Amenity with similar projects, it was not considered logistically achievable to establish the presence of and/or incorporate all neighbouring garden ponds/ornamental water features into the survey effort.

3.13 The potential presence of GCN/amphibians within neighbouring gardens and their use of garden ponds/ornamental water features (as detailed in the publication ‘Newts in your Pond and Garden’, Grundy 2007) was fully considered when assessing the predicted development effect and scale of impact and formulating appropriate mitigation and habitat enhancement measures to address the development impacts.

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4.0 SURVEY METHODS

4.1 A GCN aquatic survey and terrestrial appraisal undertaken in respect of the works should be carried out by a suitably licensed ecologist and employ a combination of recognised methodologies, as appropriate to the conditions encountered and in accordance with the survey objectives. The predicted development effect and scale of impact on GCN and other amphibians and their breeding, shelter, foraging and dispersal habitat should be assessed and appropriate mitigation and habitat enhancement measures, to address the development impacts, formulated as part of the survey effort.

4.2 The English Nature Great Crested Newt Mitigation Guidelines 2001 (EN GCN MG) indicates that a GCN survey may be necessary to check for the presence of the species if background information on distribution suggests that they may be present. Detailed indicators include:

- Any historical records for GCN on the site, or in the general area
- A pond on or near the site (within around 500m), even if it holds water only seasonally. Note that muddy, cattle-poached, heavily vegetated or shaded ponds, ditches and temporary flooded hollows can be used by GCN
- Sites with refuges (such as piles of logs or rubble), grassland, scrub, woodland, or hedgerows within five hundred metres of a pond.

4.3 The EN GCN MG recommend that, if access permission can be obtained, a GCN presence/likely absence aquatic survey using traditional methods should consist of four visits, carried out on non-consecutive nights, in appropriate weather conditions, at the appropriate time of year (mid-March to mid-June) and should ideally include all of the water bodies identified as relevant within the survey area. At least two of the survey visits should be undertaken between mid-April and mid-May.

4.4 The currently recommended GCN presence/likely absence aquatic survey methods include egg search, torch survey, bottle trapping and hand netting. The EN GCN MG recommend that, where practicable to do so, a combination of three of the four methods should be employed at each water body. Natural England advises that a GCN 'Habitat Suitability Index' score is calculated for all

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water bodies subject to a GCN survey (refer to Appendix G: GCN Appraisal/Survey Methods; Oldham *et al* (2000)).

4.5 The EN GCN MG indicate that four visits, resulting in consistent negative findings and undertaken in accordance with EN GCN MG aquatic survey methodology using traditional survey methods, represents sufficient effort to robustly establish GCN 'likely absence' at a water body.

4.6 The EN GCN MG recommend that, where access permission can be obtained, a GCN population size class assessment aquatic survey (generally required to inform a GCN mitigation licence application) should consist of six visits, carried out on non-consecutive nights, in appropriate weather conditions, at the appropriate time of year (mid-March to mid-June) to all the water bodies within the survey area where GCN presence has been established. At least three of the visits should be undertaken between mid-April and mid-May.

4.7 The GCN population size class is derived from the peak count. This is the combined sum of the highest number of adult GCN observed or captured during the same visit. GCN population size class based on the EN GCN MG categorisations are shown below.

- Small population size class: 1 to 10 individual adult GCN
- Medium population size class: 11 to 100 individual adult GCN
- Large population size class: 100 plus individual adult GCN

4.8 However, it should be noted that establishing GCN presence at a site may only require a single visit, especially if using the relatively new method of water sampling for GCN eDNA. When GCN inhabit a pond, cells containing their DNA are continually sloughed off into the water. Samples from the water column, taken by a licensed ecologist, are later analysed in a controlled laboratory environment to investigate whether GCN have recently been present in the pond (or not). Collection of samples for GCN eDNA analysis can only be undertaken between 15th April and 30th June if they are to provide a robust negative result. Samples must be analysed using a strict eDNA screening method issued by Natural England (WC1027), (refer to Appendix H: Natural

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England Technical Advice Note for field and laboratory sampling of great crested newt (Triturus cristatus environmental DNA).

4.9 Likewise, the discovery of adult and/or sub-adult GCN/amphibians within a water body or terrestrially will confirm that they are associated with the site. Additionally, the discovery of GCN/amphibian eggs, spawn, larvae or tadpoles is sufficient to confirm that a water body is a breeding site for that species.

4.10 Where access permission to inspect a water body cannot be obtained and/or it is not possible to undertake survey effort at the appropriate time of year (mid-March to mid-June) the likely GCN population size class associated with a site can generally be reliably predicted, by an experienced GCN ecologist, using historic records and/or the local status of the species combined with an assessment of the availability and suitability of aquatic and terrestrial habitats at, and neighbouring the site.

4.11 To supplement GCN aquatic survey efforts, a combination of terrestrial refugia searches can sometimes be employed in order to establish the presence of sheltering and/or foraging GCN at the site level. Such searches should be undertaken at the appropriate time of year, during appropriate weather conditions when GCN adults and sub-adults are likely to be active in their terrestrial phases.

4.12 It should be noted that findings indicating GCN likely absence, based entirely on terrestrial survey methods, should be interpreted with caution as GCN may avoid detection during the survey period.

4.13 The Amenity 2025 GCN survey was led by Dawn Goodfellow BSc (Hons) MCIEEM (Senior Ecologist), accompanied by Mike Bazley. The survey effort deemed appropriate in respect of the works was a GCN aquatic presence/absence eDNA survey and terrestrial habitat appraisal. As part of the survey a GCN 'Habitat Suitability Index' (HSI) score (Oldham *et al*, 2000) and categorisation was also calculated for Waterbodies 1 & 2.

4.14 The survey was undertaken in accordance with the terms and conditions of a GCN Licence (Class Licence WML-CL08, Registration: No. 2015-16636-CLS-CLS). The survey effort included a review of OS and aerial mapping/imaging

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and daytime site visit/walk-over inspections of the site, by an Amenity ecologist on 16th June 2025, in order to assess the status of habitats at Waterbodies 1 & 2 and at the proposed development area.

4.15 The methods employed at Waterbodies 1 & 2 included egg-search, terrestrial refugia search and GCN eDNA water sampling and analysis for GCN presence/absence, as appropriate to the timing of the visits and the conditions encountered.

4.16 Additional information relating to the presence of GCN and other amphibians within the local area (defined by Natural England as within 10 kilometres) was sourced from Magic.gov, the presence of GCN was recorded in 2015 approximately 1km to the NE of the proposed development site.

4.17 As part of the survey effort, the predicted development effect and scale of impact on GCN/other amphibians and their breeding, shelter, foraging and dispersal habitat was assessed and appropriate amphibian reasonable avoidance measures and habitat enhancement measures were formulated.

5.0 SURVEY RESULTS

5.1 The significant GCN survey effort expended by ecological consultancies and various conservation groups/organisations in the Northwest area, since the early 1980s, has established that the local status of GCN (defined by Natural England as within 10 kilometres) can best be categorised as: frequent and widespread.

5.2 The GCN population size class associated with the majority of GCN breeding sites in the Northwest area can be typically categorised as ‘small’ to ‘medium’.

5.3 The occurrence of breeding sites supporting a ‘large’ GCN population size class is considered to be atypical and unusual. However, because of the widespread status of the species within the Northwest area it is not unusual for ‘small’ or ‘medium’ GCN populations associated with a breeding site to form part of GCN ‘meta-populations’ associated with the local area.

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5.4 The main findings of the Amenity 2025 GCN survey undertaken in relation to the area of works are detailed below:

i. Information relating to the presence of GCN and other amphibians within the local area (defined by Natural England as within 10 kilometres) was sourced from Magic.gov, GCN presence was recorded just over 1km to the NE of the proposed development site boundary.
ii. Water-body A was not considered relevant to the appraisal/survey, due to it's considerable distance/isolated location from the proposed development site and the fact that it was previously found in 2018 not to support GCN.
iii. The HSI score calculated for Waterbody 1 indicated 'below average' suitability for GCN (0.5).
iv. The HSI score calculated for Waterbody 2 indicated 'good' suitability for GCN (0.74).
v. Visual observations undertaken during the site walk-over inspection visit did not result in the discovery of any evidence of GCN/other amphibians, their eggs and/or larvae being observed at Waterbodies 1 & 2.
vi. A search of the limited terrestrial refugia at the proposed development site during the walk-over inspection visit did not result in the discovery of any sheltering GCN or other amphibians.
vii. The survey robustly established the absence of GCN at Waterbodies 1 & 2 during the 2025 survey season.
viii. At the time of the survey terrestrial habitat to be directly impacted by the works was assessed to offer GCN and/or other amphibians with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Very Good'.
ix. Consequently, it has been concluded that GCN were unlikely to be present within the area/s to be directly affected by the works.

5.5 The GCN 'Habitat Suitability Index' scores/categorisation, and GCN eDNA survey result for Waterbodies 1 & 2, is detailed in Tables 2 & 3 below:

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Table 2: HSI score/categorisation (Waterbodies 1 & 2)

	Waterbody 1	Waterbody 2
Pond Ref:		
SI1 - Location	1	1
SI2 - Pond area	0.8	0.8
SI3 - Pond drying	0.9	0.5
SI4 - Water quality	0.33	0.33
SI4 - Shade	1	1
SI6 - Fowl	0.67	0.67
SI7 - Fish	0.01	0.67
SI8 - Ponds	0.83	0.83
SI9 - Terr'l habitat	1	1
SI10 - Macrophytes	0.8	1
HSI	0.50	0.74
Categorisation	**Below Average**	**Good**

NB: <0.5 poor, 0.5-0.59 below average, 0.6-0.69 average, 0.7-0.79 good, >0.8 excellent

Table 3 (refer to Appendix I: GCN eDNA Analysis for full report):

TECHNICAL REPORT						
ANALYSIS OF ENVIRONMENTAL DNA IN POND WATER FOR THE DETECTION OF GREAT CRESTED NEWTS						
Folio No.:	2534-2025					
Matters Affecting Results:	None					
Results:						
Lab Sample No.:	**Site Name:**	**OS Ref:**	**Degradation Check:**	**Inhibition Check:**	**Result:**	**Positive Replicates:**
R25/1092	Secret Lake, W1	SJ 88735 94620	Pass	Pass	Negative	0/12

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TECHNICAL REPORT						
ANALYSIS OF ENVIRONMENTAL DNA IN POND WATER FOR THE DETECTION OF GREAT CRESTED NEWTS						
Folio No.:	2535-2025					
Matters Affecting Results:	None					
Results:						
Lab Sample No.:	**Site Name:**	**OS Ref:**	**Degradation Check:**	**Inhibition Check:**	**Result:**	**Positive Replicates:**
R25/1093	Secret Lake, W2	SJ 89039 94711	Pass	Pass	Negative	0/12

5.6 Based on the survey findings it was concluded that sufficient information was available to allow the predicted development effect and scale of impact to be assessed.

6.0 PREDICTED DEVELOPMENT EFFECT & SCALE OF IMPACT

6.1 As Waterbodies 1 & 2 are situated within the proposed development site it is possible that they may be affected by the development, (though likely this would be Waterbody 2 only). However, it is considered that Waterbody 2 sometimes dries so is unlikely to support amphibian breeding every year.

6.2 The proposed works are likely to result in some; partial destruction, modified management/resurfacing, etc., temporary disturbance/destruction and reinstatement of immediate (<50 metres), intermediate (>50 metres to <250 metres) and distant (>250 metres) terrestrial habitat associated with Water-bodies 1 & 2 and/or other ponds/waterbodies in the local area (defined by Natural England as within 10 kilometres).

6.3 The proposed works are unlikely to increase the historic and existing level of habitat fragmentation at the site level and will not isolate any GCN population/s or adversely impact on their ability to migrate/disperse and reach their breeding sites.

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6.4 The development proposals for the site could increase (from current and historic levels) the risk of post-development human interference impacts on any GCN population/s and/or aquatic and terrestrial habitats associated with the local area.

6.5 The predicted development effect and scale of impact have been assessed, with reference to the English Nature Great Crested Newt Mitigation Guidelines 2001 and, in the absence of appropriate mitigation measures, a 'potentially medium' negative development impact was predicted for any GCN/amphibian populations potentially associated with the local area.

6.6 The predicted development effect and impacts are detailed/highlighted (blue) in Table 4 below (adapted from the EN GCN MG).

Table 4: Summarising the scale of main impacts on GCN at the site level

Habitat Feature	Development Effect	Scale of Impact		
		Low	Medium	High
Confirmed GCN breeding pond/water body	Destruction			✓
	Isolation caused by fragmentation			✓
	Partial destruction; modification		✓	
	Temporary disturbance	✓		
	Post-development interference			✓
Other pond or water body	Destruction		✓	
	Isolation caused by fragmentation		✓	
	Partial destruction; modification	✓		
	Temporary disturbance	✓		
	Post-development interference	✓		
Immediate Terrestrial Habitat (less than 50 metres from a GCN/breeding pond or other water body potentially used for breeding by the species/other amphibians)	Destruction			✓
	Isolation caused by fragmentation			✓
	Partial destruction		✓	
	Modified management, resurfacing etc.		✓	
	Temporary disturbance	✓		
	Post-development interference		✓	
	Temporary destruction & reinstatement	✓		
Intermediate Terrestrial Habitat: (50m – 250m from a GCN/breeding pond or other water body potentially used for breeding by the species/other amphibians)	Destruction		✓	
	Isolation caused by fragmentation		✓	
	Partial destruction	✓		
	Modified management, resurfacing, etc.	✓		
	Temporary disturbance	✓		
	Post-development interference	✓		
	Temporary destruction & reinstatement	✓		
Distant Terrestrial Habitat (more than 250 metres from a GCN/breeding pond or other water body potentially used for breeding by the species/other amphibians)	Destruction	✓		
	Isolation caused by fragmentation	✓		
	Partial destruction	✓		
	Modified management, resurfacing etc.	✓		
	Temporary disturbance	✓		
	Post-development interference	✓		
	Temporary destruction & reinstatement	✓		

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6.7 Based on the survey findings, it has been concluded that GCN are highly unlikely to be present at the proposed development site. Consequently, the proposed development is considered very unlikely to result in a breach of current wildlife legislation and/or Natural England guidance relating to the species and its shelter habitat.

6.8 Given that the proposed development will not have a significant or adverse impact on the favourable conservation status of any known GCN populations it has been concluded that GCN are unlikely to pose a constraint to the proposed development.

6.9 However, it is considered that the development could potentially have an adverse impact on any amphibians not encountered during the survey (e.g. common frog, smooth newt, and/or common toad), should they inadvertently stray onto the development site following the commencement of development and take cover under/within shelter habitat incidentally created as a consequence of the proposed development works, i.e. areas of disturbed ground, excavations, spoil mounds and/or stored materials.

6.10 Whilst more common amphibians do not have the same legal protection as GCN, it is considered appropriate to recommend a mitigation strategy involving the implementation of amphibian Reasonable Avoidance Measures (RAM) in order to reduce potential development impacts on these species and to enhance the site for these species post-development, (in line with NPPF).

7.0 RECOMMENDED REASONABLE AVOIDANCE MEASURES & ENHANCEMENT STRATEGY

7.1 The careful implementation of the RAM detailed below will proportionately and appropriately address the possible impact of the proposed development works on any amphibian populations potentially associated with Waterbodies 1 & 2 and/or in the wider area.

1. The winter hibernation/dormancy period for amphibians is generally accepted to run from November to February/March, with hibernation/dormancy for individual amphibians usually commencing following the first hard winter frost. Consequently, any work which is likely to result in the disturbance of a potential

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amphibian hibernation site should not be scheduled or undertaken during this period or during any periods of cold or frosty weather outside of this period when amphibians are likely to be hibernating/dormant. Should such works be required the Appointed Ecologist will formulate and implement appropriate measures to minimise the risks to hibernating amphibians.

2. Where practicable, the duration/timing of the enhancement works, and implementation of RAM should encompass the period when the majority of amphibians are considered likely to be at their breeding sites. Consequently, the risk of amphibians being adversely impacted by the enhancement works will be minimised.

3. Prior to commencing the enhancement works at the site a suitably experienced Consultant Ecologist should first be appointed by the developer/contractor to ensure that the RAM are carefully followed.

4. The RAM will be implemented as deemed appropriate by the Appointed Ecologist. A copy of this report and supporting materials such as 'Amphibian ID Sheets' and 'on call' Ecologist contact details should be kept on the site and be available to refer to during the site enhancement works.

5. Prior to any site enhancement works starting the developer/contractors should attend an ecological site induction, where the implications arising from the presence of amphibians in the local area and the need to implement the RAM detailed in this report should be fully detailed by the Appointed Ecologist.

6. Prior to any enhancement works starting at the site, the extent of the works should be agreed between the contractors/developer and the Appointed Ecologist.

7. The developer/contractors and the Appointed Ecologist should work together to ensure that no areas of aquatic or terrestrial habitat to be retained at/adjacent/neighbouring the site are lost or damaged as a consequence of the enhancement works.

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Discovering Amphibians On-Site

8. If at all possible, amphibian capture should always be carried out by the Appointed Ecologist or their Accredited Agent.

9. However, in the event that ecological supervision is not possible on-site when an amphibian (other than GCN) is found terrestrially the developer/contractor should place the amphibian/s into a secure, clean container/s lined with damp moss. If more than one amphibian species is found (other than GCN) they should be kept in separate secure, clean container/s lined with damp moss to avoid injury and/or predation. The Appointed Ecologist or their Accredited Agent should be contacted immediately (using the 'on call' service, if appropriate) to ensure that the amphibian species is correctly identified/recorded and for advice on where and how to release it (as deemed appropriate by the Appointed Ecologist).

10. **The amphibian RAM proposed does not allow GCN to be captured or removed from the site and released at another location (as personnel require a survey licence to handle GCN). In the highly unlikely event that GCN are found within the working area works will cease and the Appointed Ecologist immediately contacted for advice on how best to proceed.**

7.2 Proposed Habitat Enhancement Measures:

7.2.1 A log refugia pile should be created alongside a habitat edge near Waterbody 1 to provide suitable amphibian refuge/hibernation habitat. The log refugia pile should be a minimum of 1m long x 1m wide x 0.5m high and topped with soil, which is later seeded (using a grassland seed mix).

7.2.2 Where boundary hedgerows already exist, these should preferably be retained post-development, wherever possible. Any gaps in these areas of hedgerow should be infilled by planting tree whips of native hedgerows tree species (preferably of local provenance).

7.3 Future habitat creation/enhancement/management works at or neighbouring the site will be informed by current best practice relating to the long-term

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management of amphibian aquatic and terrestrial habitats as detailed in the Amphibian Habitat Management Handbook.

7.4 Appropriate resources will be made available by the developer/landowner to ensure that the required habitat enhancement works at the site are successfully delivered.

8.0 CONCLUSIONS

8.1 Based on the survey findings it has been concluded that GCN are unlikely to be present at the proposed development site. Consequently, the proposed development is considered unlikely to result in a breach of current wildlife legislation and/or Natural England guidance relating to the species and its shelter habitat.

8.2 Given that the proposed development will not have a significant or adverse impact on the favourable conservation status of any known GCN populations it has been concluded that GCN are unlikely to pose a constraint to the proposed development.

8.3 However, it is considered that the development could potentially have an adverse impact on any amphibians not encountered during the survey, should they inadvertently stray onto the development site following the commencement of development and take cover under/within shelter habitat incidentally created as a consequence of the proposed development works, i.e. areas of disturbed ground, excavations, spoil mounds and/or stored materials.

8.4 Whilst more common amphibians do not have the same legal protection as GCN, it is considered appropriate to recommend a mitigation strategy involving the implementation of amphibian Reasonable Avoidance Measures (RAM) in order to reduce potential development impacts on these species and to enhance the site for these species post-development, (in line with NPPF).

8.5 The predicted development effect and scale of impact have been assessed, and appropriate reasonable avoidance measures and enhancement measures

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have been formulated. It should therefore be possible for the Local Planning Authority to make an informed decision as to whether it is possible to discharge their responsibilities under current planning guidance, in respect of GCN/amphibians and biodiversity, by the implementation of planning condition/s when determining the planning application submitted in respect of the works.

9.0 REFERENCES

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APPENDICES

Appendix A: Senior Ecologist's Experience

Dawn Goodfellow BSc (Hons) MCIEEM is a Senior Ecologist, working with Amenity Ltd. She is licensed to survey for great crested newts in both England and Wales and works alongside a dedicated team of motivated, professional and highly experienced ecologists and field team workers, with the focus on the delivery of quality survey reports and, where required, mitigation for protected species for projects across northern England and North Wales. She holds a Natural England GCN survey class licence WML-CL08 Reg No. 2015-16636-CLS-CLS, a Natural Resources Wales GCN survey licence (Ref No.: 57238:OTH:SA:2014) and has recently been the named Appointed Ecologist on three Natural England and/or Natural Resources Wales GCN mitigation licences.

Dawn has been involved with a large number of GCN and amphibian-related projects in various capacities and roles since 2001. She is fully conversant with current wildlife legislation relating to GCN and other protected species and the responsibilities of Local Planning Authorities in respect of protected species and biodiversity. As a professional ecologist and Full Member of the Chartered Institute of Ecology and Environmental Management (CIEEM) she adheres to their Code of Professional Conduct. She regularly employs the methodologies detailed in the English Nature Great Crested Newt Mitigation Guidelines, 2001 (EN GCN MG). Dawn also has extensive experience of the Local Authority planning process and the Natural England and Natural Resources Wales GCN/protected species licensing systems and has developed good working relationships with Local Authority Officers and Natural England and Natural Resources Wales protected species licensing advisors. She has also acted as expert witness (Appellant) and produced supporting evidence in respect of ecology for a successful planning appeal.

Since early 2014 Dawn has been instrumental in developing GCN Appraisal reports for use on ‘low’ impact developments with regard to GCN, i.e. those not considered likely to have a significant effect on the favourable conservation status of GCN. In this capacity Dawn helped to design appropriate mitigation and habitat compensation measures to address ‘low’ development impacts. Where deemed appropriate, and in accordance with current Natural England and Natural Resources Wales GCN/protected species guidelines and standing advice, Dawn takes a considered and proportionate approach to the formulation and implementation of GCN/protected species mitigation measures. As part of a wider team Dawn has successfully delivered to date over 60 non-licensed (Reasonable Avoidance Measures) GCN mitigation strategies in respect of development-related projects.

In 2016 Dawn undertook CIEEM training to learn how to properly undertake water sampling for GCN eDNA Analysis.

Appendix B: GCN & Amphibian Legislation

Species/Habitat	Protected by:	UK BAP
Common frog	Provision 5 of Section 9 of the *Wildlife and Countryside Act*, 1981 (as amended)	No
Common toad	Provision 5 of Section 9 of the *Wildlife and Countryside Act*, 1981 (as amended) Section 41 of the *Natural Environment and Rural Communities (NERC) Act,* 2006	Yes
Great crested newt	Regulation 41 of *The Conservation of Habitats and Species (Amendment) Regulations*, 2012 Section 9 of the *Wildlife and Countryside Act,* 1981 (as amended) Section 41 of the *Natural Environment and Rural Communities (NERC) Act,* 2006	Yes
Smooth newt	Provision 5 of Section 9 of the *Wildlife and Countryside Act*, 1981 (as amended)	No

The *Conservation of Habitats and Species Regulations,* 2010

European protected species are listed on Schedule 2 of the *Conservation of Habitats and Species Regulations* 2010. Those species listed on Schedule 2 are protected under Regulation 41, which refers to the protection of wild animals of a European Protected Species. The following is a summary of the offences listed under Regulation 41, however, the *Conservation Regulations* should always be referred to for the exact and current wording:

Under Regulation 41 of the *Conservation of Habitats and Species Regulations,* 2010 it is an offence to –

- deliberately capture or kill a wild animal of a European protected species;
- deliberately disturb wild animals, in particular any disturbance which is likely:
  - to impair their ability to survive, to breed or reproduce, or to rear or nurture their young; or
  - to impair their ability, in the case of animals of a hibernating or migratory species, to hibernate or migrate;
  - to affect significantly the local distribution or abundance of the species to which they belong
- deliberately take or destroy the eggs of such an animal; or
- damage or destroy a breeding site or resting place of such an animal.
- keep, transport, sell or exchange, or offer for sale or exchange, any live or dead wild animal of a European protected species, or any part of, or anything derived from, such an animal.

Wildlife and Countryside Act, 1981 (as amended)

British protected species of animal are listed on Schedule 5 of the *Wildlife and Countryside Act*, 1981 (as amended). Those species listed on Schedule 5 are protected under Part 1, Section 9, which refers to the protection of certain wild animals. The following is a summary of the

offences listed under Section 9; however the Act should always be referred to for the exact and current wording:

Under Section 9 of the *Wildlife and Countryside Act*, 1981 (as amended) if any person –

- intentionally kills, injures or takes any wild animal included in Schedule 5;
- has in his possession or control any live or dead wild animal included in Schedule 5 or any part of, or anything derived from such an animal;
- intentionally or recklessly damages or destroys, or obstructs access to, any structure or place which any wild animal included in Schedule 5 uses for shelter or protection;
- disturbs any such animal included in Schedule 5 while it is occupying a structure or place which it uses for that purpose;
- sells, offers or exposes for sale, or has in his possession or transports for the purpose of sale, any live or dead wild animal included in Schedule 5, or any part of, or anything derived from, such an animal; or,
- publishes or causes to be published any advertisement likely to be understood as conveying that he buys or sells, or intends to buy or sell, any of those things, he shall be guilty of an offence.

This legislation applies to all life stages of GCN. Heavy fines (up to £5,000 per incident) can be imposed for **each** offence, and a prison sentence of up to 6 months for each offence can be given to any person found guilty of an offence. In certain circumstances, any machine, tool or implement involved in an illegal act can also be seized.

Natural England (NE) issue licences for the disturbance of European Protected Species including GCN, certain criteria must be met before a licence can be issued to enable otherwise prohibited works to proceed. Such criteria may be subject to change without notice. For further information please visit www.naturalengland.org.uk

The *Natural Environment and Rural Communities (NERC) Act,* 2006 (as amended)

The following is a summary of the *Natural Environment and Rural Communities (NERC),* Act, 2006 (as amended), the *NERC Act* itself should be referred to for the exact and current wording:

- Every public authority must, in exercising its functions, have regard, so far as is consistent with the proper exercise of those functions, to the purpose of conserving biodiversity;
- In complying with the above, a Minister of the Crown, government department or the National Assembly for Wales must in particular have regard to the United Nations Environmental Programme Convention on Biological Diversity of 1992;
- Conserving biodiversity includes, in relation to a living organism or type of habitat, restoring or enhancing a population or habitat.

Appendix C: Natural England GCN Standing Advice Sheet Flow Chart

Guidance on how to assess a great crested newt survey and mitigation strategy

(The numbers in each box are to assist in referencing a decision trail rather than being a numerical sequence through the flow chart)

1. **START**
Is the application within/close to a SSSI or SAC notified for great crested newts? (See: www.magic.gov.uk)

Yes → 2. Consult Natural England on the potential implications for the site and/or any agreed planning and survey protocols or guidance for the site(s)

No → 5. Does the survey report identify suitable features on or adjacent to the application site for newts eg ponds within 500m, hibernation sites, foraging habitat, commuting corridors?

No → 3. Accept findings and consider promoting biodiversity enhancements for great crested newts (eg creation of new water bodies and suitable terrestrial habitat) in accordance with the NPPF and Section 40 of the NERC Act. **END**

Yes → 6. Has the assessment of habitats and impacts been undertaken to the satisfaction of the LPA and in accordance with Great Crested Newt Mitigation Guidelines?

No → 8. Further survey effort required in accordance with Great Crested Newt Mitigation Guidelines – request additional information from applicant and ask Question in Box 6 again. If not provided, then application should be refused. **END**

Yes → 7. Has the applicant confirmed that effects on great crested newt can be avoided AND has this been confirmed by specialist advice to the satisfaction of the LPA?

Yes → 4. Accept the findings. Issue an informative regarding appropriate action to be taken if great crested newts are encountered during development, Secure working methods and programme using conditions where necessary to avoid impacts. Consider encouraging biodiversity enhancements great crested newts (e.g. creation of habitat linkages) in accordance with NPPF and Section 40 of the NERC Act. Permission can be granted. **END**

No → 9. Has a detailed newt survey been carried out at the right time of year, using sufficient survey techniques and effort and over adequate search area?

No → 8. Further survey effort required in accordance with Great Crested Newt Mitigation Guidelines – request additional information from applicant and ask Question in Box 6 again. If not provided, then application should be refused. **END**

Yes → 10. Have great crested newts been found on the application site/in the study area?

No → 11. Are effects on great crested newts or their habitats likely?

No → 18. If no go to **box 6**

Yes → 11. Are effects on great crested newts or their habitats likely?

Yes → 14. Is mitigation proposed that will:
- ensure no net loss of habitat in terms of quantity and quality
- maintain habitat links
- secure long-term management of the site for benefit of newts
- ensure receptor sites (where required) are within the home range of the existing population and free from future development threats.

No → 17. Request further or more appropriate mitigation/compensation and consider question in Box 14 again. If applicant fails to provide satisfactory information, consider refusal of Application.

Yes → 13. When mitigation is taken into account, are the proposals likely to result in offences under Regulation 41(1) of the Conservation of Habitats and Species Regulations 2010 (as amended) (Article 12(1) of The Habitats Directive 92/43/EEC) and require a derogation licence?

No → 16. Permission may be granted subject to appropriate planning conditions and obligations. **END**

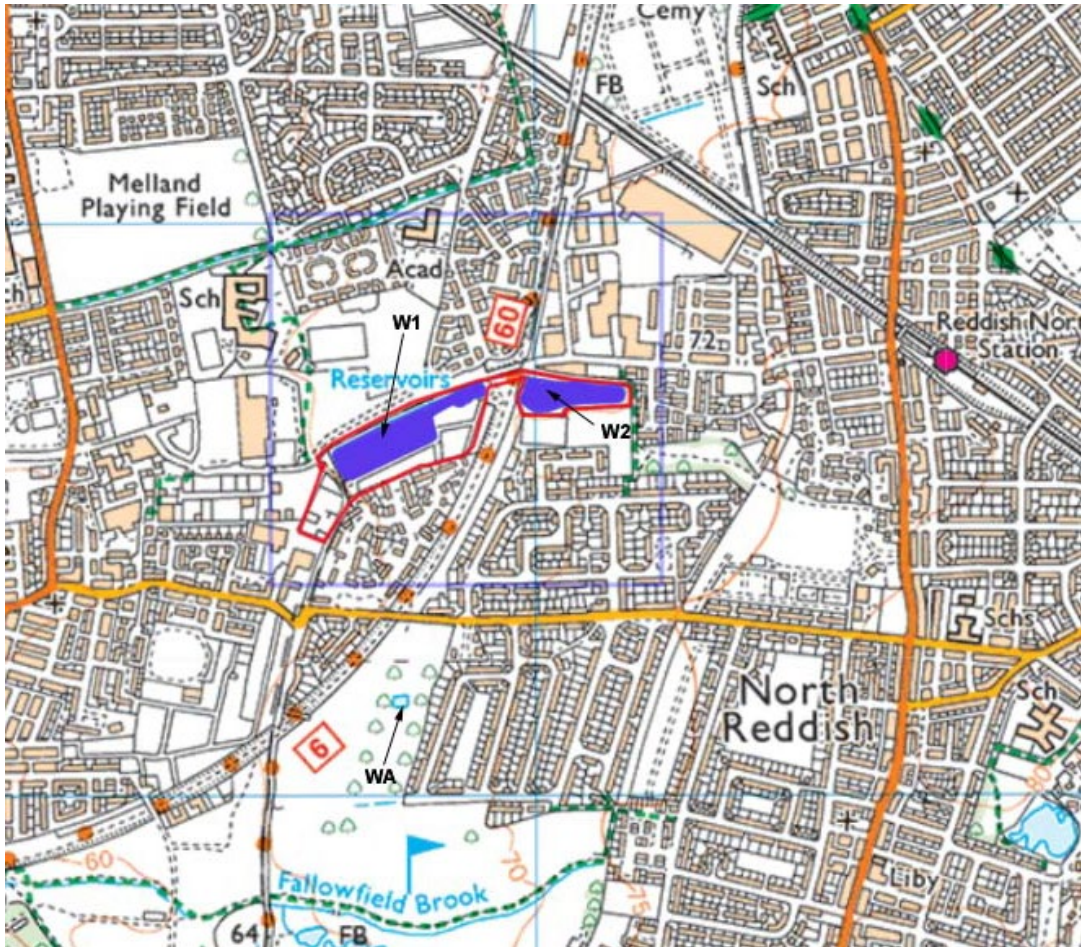
Yes → 15. After considering the three legal tests*, does the LPA consider that Natural England is likely to refuse a licence?

Yes → 12. Application should be refused as it does not comply with the Conservation of Habitats and Species Regulations 2010. **END**

No → 16. Permission may be granted subject to appropriate planning conditions and obligations. **END**

*** See also:** https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/82706/habitats-simplify-guide-draft-20121211.pdf

Appendix D: Site Location Plan (OS)



Key:

- Proposed development site boundary
- Numbered water-bodies located on or within 250 metres of the proposed development site boundary
- Lettered water-bodies located 250 - 500 metres from the boundary of the proposed development site

Appendix E: Photographic Plates

Plate 1: Water-body 1 comprises a large fishing lake situated in-between residential and amenity areas, it was found not to support GCN, as proven by the GCN eDNA analysis

Plate 2: Water-body 2 was mainly vegetated by common reed and was considered to dry out sometimes, it was situated in-between residential and amenity areas and was found not to support GCN, as proven by the GCN eDNA analysis

Appendix F: Land HSI (LHSI)

Suitability for GCN	GCN Habitat Type	Example Areas
Very Poor	No GCN hibernation/shelter potential; No habitat suitable for GCN foraging; No connectivity to suitable habitat; and Complete barriers to GCN dispersal.	Large areas of intact hard standing Very compacted amenity grass, e.g. football pitches Built-up areas Commercial/industrial areas
Poor	No GCN hibernation/shelter potential; Limited habitat for GCN foraging; Limited connectivity to suitable GCN habitat; and Significant restrictions/barriers present to limit the dispersal of GCN.	Compacted bare ground/hard-standing Commercial/industrial areas Intensively managed agricultural land Closely mown amenity grassland School playing field
Below Average	Sites with limited availability of GCN shelter sites; No obvious GCN hibernation sites; Limited GCN foraging habitat present; Limited connectivity to GCN habitat; and Limited GCN dispersal habitat present.	Managed arable farmland High density grazing Grassland that is intensively managed Formal planting Commercial/industrial sites
Average	Some hibernation/shelter sites; Some habitat available for foraging; Some connectivity to suitable habitat; and Some barriers/restrictions to GCN dispersal.	Semi-improved grassland with regular management Grazing land Grassland with limited management Hedgerows with narrow buffer zones Brownfield sites abandoned <5yrs previously Gardens
Above Average	Land with hibernation and shelter sites; Varied habitat for foraging; Linked/connected habitats; and Few barriers present to limit GCN dispersal.	Unimproved grassland Unmanaged semi-improved grassland Mature hedgerows with wide buffer zones Low density grazed pasture Mature gardens Brownfield sites abandoned >5yrs previously
Good	Land with a range of hibernation and shelter sites; Varied and diverse habitat for foraging; Habitats with connectivity to areas suitable for GCN; and Few/no barriers present to limit GCN dispersal.	Grassland that is rough/tussocky Dense scrub Newly-planted woodland Organic managed farmland Railway embankments Allotments Mineral workings
Very Good	Widespread hibernation and shelter sites; Extensive varied and diverse foraging habitat; Large areas that are well-connected; No barriers present to GCN dispersal.	Mature broad-leaved woodland Managed wildlife areas/reserves Old mineral workings/quarries

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Appendix G: GCN Appraisal/Survey Methods

Appraisal/Survey Methods for Aquatic Habitats

GCN eDNA analysis: When GCN inhabit a pond, cells containing their DNA are continually sloughed off into the water. Samples from the water column, taken by a licensed ecologist, are later analysed in a controlled laboratory environment to investigate whether GCN have recently been present in the pond (GCN positive) or not (GCN negative). Collection of samples for GCN eDNA analysis can only be undertaken between 15th April and 30th June if they are to provide a robust result. Samples must be analysed using a strict eDNA screening method issued by Natural England (WC1027).

Egg-search: All native newts lay their eggs one-by-one on submerged leaves of aquatic vegetation and other suitable substrates that are submerged. Careful inspection of aquatic vegetation can therefore be used to confirm the presence of newts in a pond during the amphibian breeding season. GCN eggs can be differentiated from those of the smaller UK native newts (i.e. smooth and palmate newts) by their size and colour, enabling species-specific presence to be confirmed. Where GCN eggs have been identified (establishing that the pond as a breeding site) no further egg-search effort need be undertaken at that pond, as additional disturbance could result in egg predation and/or reduce viability of the eggs.

Torch-survey: Using 'Clulite' 1-million candlepower torches, this is a robust method of establishing newt presence and to count the number of newts in ponds during the breeding season. Weather conditions can limit surveying; dry, still nights are required to allow effective observation, as can the condition of the pond, i.e. deep, weedy or turbid ponds may require additional survey methods to be utilised.

Bottle-trapping: This method is useful where ponds/water is turbid or where an abundance of vegetation would restrict torch-survey. It can assist in confirming newt presence and to indicate population size. Ideally, traps are set at 2 metre spacings around the margins of the pond, where it is possible to set traps, during the evening and these are collected the following morning. This method allows accurate identification to species, sex and life stage. Where some areas of the pond may be inaccessible; i.e. due to steep sides, the presence of dense vegetation, deep water/silt or overhanging trees, etc., the bottle traps can occasionally need to be set in random transects, utilising, wherever possible, the same number of traps as would be required for the 'ideal' 2 metre spacings. However, bottle-trapping should not be used in areas where there are health and/or safety concerns, i.e. steep banks and deep water/silt, or where there is a possible risk of disturbance from animals or interference from vandals or other members of the public.

Netting: This method can be useful in confirming GCN presence within a pond (especially small, garden ponds); however, it should not be regarded as particularly reliable because adult GCN and their larvae may evade capture, especially in deep, large or thickly vegetated ponds (as GCN prefer to swim in open water).

Habitat Suitability Index: As part of all GCN surveys, a 'Habitat Suitability Index' (HSI) score should be calculated for each pond included in a survey. The HSI score is a measure of GCN habitat suitability and can be used to help determine the likelihood of GCN presence within a pond. The HSI score is determined by assessing 10 environmental factors, i.e. the likely presence of fish, water fowl, water quality, etc. The results obtained are converted to a figure and this is calculated to provide a score between 0 and 1. This score can then be

used to categorise GCN aquatic/pond habitat (with 0 representing unsuitable and 1 optimal habitat). However, the system is not sufficiently exact to be able to conclude that a pond with a high score will support GCN, or that any pond with a low score will not support GCN.

It is not always physically possible to utilise three or more survey methods at each pond, this is because ponds frequently differ in terms of water depth, surface area, and water quality. Constraining factors such as steep sides, deep water/silt, the risk of bottle traps being disturbed or damaged by livestock or the public, or torch-survey being restricted by turbid or weedy ponds, determine which methods can be safely and effectively used to provide results that can be considered robust.

Appraisal/Survey Methods for Terrestrial Habitats

Hand-searches of Terrestrial Refugia

Hand-searches of terrestrial refugia is only recommended by natural England for distinct habitat features, i.e. natural refugia such as log piles and/or artificial refugia, that can be carefully lifted/dismantled by hand with very little risk of harm to GCN and/or other amphibians. Refugia searches should not be undertaken when GCN/amphibians are inactive (i.e. November to February inclusive) or in extremely hot periods in summer. Searches should only be carried out in suitable weather conditions as detailed in the English Nature Great Crested Newt Mitigation Guidelines (EN GCN MG, 2001).

Night-time Torch-searches of Terrestrial Habitats

The use of terrestrial torch-searches for foraging and/or migrating GCN/amphibians is only recommended by Natural England to be used by highly experienced amphibian ecologist/s using high powered hand-torches (with a minimum of 1 million candle power). The searches should only be undertaken on mild nights either during rainfall, or shortly after rain, when GCN/amphibian prey species such as earthworms and slugs are active above ground. The search effort should involve repeated scanning of all areas to look for GCN/amphibians emerging from the ground with checks made at ground level along linear features such as amphibian fence lines and/or exterior walls of buildings. The ecologist should walk slowly scanning their torch in front and checking suitable amphibian refuges. The search should cease if much leaf fall occurs as this makes GCN/amphibians difficult to find and there is a danger of treading on GCN and/or other amphibians.

Appendix H: Natural England Technical Advice Note for field and laboratory sampling of great crested newt (Triturus cristatus) environmental DNA

Analytical and methodological development for improved surveillance of the Great Crested Newt

WC1067

Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (*Triturus cristatus*) environmental DNA

This report should be cited as:
Biggs J, Ewald N, Valentini A, Gaboriaud C, Griffiths RA, Foster J, Wilkinson J, Arnett A, Williams P and Dunn F 2014. Analytical and methodological development for improved surveillance of the Great Crested Newt. Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (*Triturus cristatus*) environmental DNA. Freshwater Habitats Trust, Oxford.

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1. Scope of document

Environmental DNA (eDNA) is nuclear or mitochondrial DNA that is released from an organism into the environment. Sources of eDNA include secreted faeces, mucous, and gametes; shed skin and hair; and carcasses. In aquatic environments, eDNA is diluted and distributed in the water where it persists for 7–21 days, according to the detection limits of qPCR approaches and associated fragment sizes, and depending on environmental conditions (Biggs et al. 2014). Recent research has shown that the DNA from a range of aquatic organisms can be detected in water samples at very low concentrations using qPCR (quantitative Polymerase Chain Reaction) methods.

This document provides technical advice for laboratories and field staff collecting and analysing samples for qPCR analysis of great crested newt (*Triturus cristatus*) environmental DNA. The document:

- Sets out the standards required
- Sets out field and laboratory approaches for screening the presence/absence of the great crested newts
- Is designed to deliver a consistent approach, and hence comparable data, between laboratories for use in decision making.

Deviations from this protocol will need to demonstrate equivalence.

This document is based mainly on research undertaken during Defra project WC1067 "Analytical and methodological development for improved surveillance of the great crested newt" (Biggs et al. 2014). We advise that this report is used as a reference document for those carrying out great crested newt surveys using the methods described here.

2. Quality assurance and quality control

The methods described in this technical advice note are designed to reduce as far as possible the risk of field or laboratory generated false positive and false negative results. Quality control measures must be extended to sample collection, preservation and handling, as well as laboratory protocols, since assurances of sample quality will prove critically important to the avoidance of false negatives.

The field of aquatic eDNA is developing rapidly and it is likely that, as methods evolve, appropriate updates will need to be made to the processes detailed in this technical advice note.

2.1 Laboratory standard

Laboratories undertaking eDNA analysis should be able to demonstrate adequate quality assurance standards. Typically these will comprise a documented quality management system which would usually follow, or be equivalent to, the outline of ISO/IEC 17025 standard.

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

In the meantime, agencies and organisations may wish to include samples from ponds known to support great crested newt and samples from sites known to be free from great crested newts to validate sampling programmes.

2.2 Sample acceptance

The laboratory analysing eDNA water samples should have a standard and documented sample acceptance procedure in place. This should include:

- Date and time of sample receipt
- Sample condition (i.e. has the sample container been damaged in any way)
- A visual verification of the sample volume (to detect any leakages)
- A note to confirm appropriate handling in transit (e.g. courier packaging intact).

The receiving laboratory should transfer the sample number to the sample acceptance record at this point.

2.3 Stability of field sampling kits

The stability of field sampling kits should be assessed through the use of an appropriate artificial DNA marker to check for unexpected decay of DNA between sampling and sample analysis. Details of the marker used, expected rates of decay and actual decay rates should be published alongside eDNA results for the target species. The marker can be chosen by the laboratory or the marker used in WC1067 can be purchased from Spygen.

2.4 Outcome required

Biggs et al. (2014) achieved a Limit of Quantification of $3 * 10^{-3}$ ng/L: at present there is no evidence that great crested newt eDNA can be quantified with precision and accuracy below this level. Failure to achieve detection at this limit will lead to increased risk of false negative results for sites where great crested newt occur at low density. There should be no detection of closely related species. In the case of the great crested newt in the UK, the risk is mainly of detecting the Italian crested newt (*Triturus carnifex*) which is present at a few of locations. The primers and probe were also tested on tissue samples of marbled newt (*Triturus marmoratus*). None of these samples were amplified, confirming the suitability of the primer pair and probe for the great crested newt. The primers and probe also did not bind with the DNA of other UK native newts (smooth and palmate newt) which are in the genus *Lissotriton*.

2.5 Identification of risks of false positives and false negatives

There are risks of both false positives and false negatives in eDNA analysis (Darling and Mahon, 2011). Errors can occur in both field and laboratory stages of the work. Given the test's sensitivity it appears that the main risk from contamination will be from false positives.

The main risks, and their mitigation for great crested newt eDNA work, are:

(i) Molecular assay design: mitigated in research and development phase of primer and probe design. Salt free primers should be used. The quality of the primer and of the PCR mix is assured by the standards.

(ii) Laboratory quality control: mitigated by laboratory design and process control.

(iii) Sampling design: mitigated by site selection procedures in field monitoring programmes.

(iv) Uncertainty in the relationship between presence of target DNA and presence of viable target organisms: mitigated partially by research so far undertaken, and by future research increasing knowledge of great crested newt eDNA.

Table 1 summarises information on situations which may have an increased risk of generating false negatives and false positives, and potential ways to mitigate these risks. For the field sampling protocol, the risk of contamination may be greater for specialist contractors undertaking large numbers of great crested newt surveys compared to volunteers making infrequent visits to a small number of sites.

Table 1. Risk, and mitigation, of false positives and false negatives

Risk factor	Mitigation
Field-based false positives	
Cross contamination between sites (due to equipment, clothing etc.).	Ensure that there is no contact between contaminated material and the water being preserved in the sampling process.
Inflows, bringing eDNA from sites with newts into unoccupied ponds. Note that there is so far little evidence that this is a significant problem but it is a theoretical possibility.	This risk cannot be eliminated at present and its extent is not understood. Where ponds have inflows, survey teams will have to make judgements about the likely impact of any inflow. However, the majority of great crested newt ponds lack substantial inflows. The presence/absence of inflows, and whether they are wet or dry at the time of survey should be recorded in field notes.
Aquatic animals (e.g. herons, water voles) transferring newt DNA between sites (e.g. in faeces, in water trapped in fur)	This risk cannot be eliminated and the extent to which it occurs is currently unknown. Further research will be required to assess whether this is a significant risk, although at present it seems likely to be small.
Field-based false negatives	
Low numbers of newts	This risk is minimised by following good field protocol. Note that at present the minimum number of newts that can be detected in different waterbodies is not known. However, ponds with torch counts of zero animals in the breeding season, where newts were known to be present, have provided positive eDNA results in the breeding season.
Very wide, shallow drawdown zones may increase the likelihood of collecting water samples in areas where there has been no newt activity even though the pond is currently occupied.	To access deeper water areas it is possible that the water sampler could be added to a long pole. It is important not to enter the water as sediments will be disturbed which may contain historical great crested newt DNA. Further research data on sediment DNA is likely to be available within 6-12 months to refine understanding of this issue. In all water depths it is necessary to gently stir the water throughout its depth, without disturbing sediments, as eDNA is believed to sink. It is advisable to avoid sampling very shallow water (less than 5-10 cm deep) as it may be difficult to avoid stirring up sediment in these areas.
There is evidence that DNA is less likely to be detected in water taken from densely packed mats of vegetation; either because of a lack of newt activity or because of the difficulty of sample collection in these areas.	Avoid sampling in these areas: sample from water in areas where vegetation is suitable for egg-laying and open water areas suitable for displaying.
There is evidence that eDNA is less likely to be detected if the whole pond perimeter is not sampled.	Every effort should be made to access 20 sites around the pond for sampling. Sites where 80-90% of pond margins were accessed achieved 99.3% detection rates. Attaching the sampling ladle to an extension pole may be an option for reaching a wider range of areas. Effective cleaning of the extension pole between sites is essential. The pole must be kept separate from any equipment that is in contact with newts.

Table 1 (cont). Risk, and mitigation, of false positives and false negatives

Risk factor	Mitigation
Laboratory false positives	
Contamination of eDNA sampling kits.	Mitigation is largely ensured by good laboratory design, set-up and processes, particularly separation of the sample preparation room from all other stages of the process.
Contamination during DNA amplification.	Mitigation is largely ensured by physical separation of the different stages of the PCR process, use of dedicated equipment and lab coats for each stage and a uni-directional work flow from clean to DNA contaminated rooms.
The risk of contamination in the laboratory is likely to be greatest when larger numbers of samples (>20) and multiple batches of samples are handled.	Mitigation is largely ensured by good laboratory design, set-up and processes. It is to be expected that handing of smaller batches of samples (i.e. <20 samples), in single trials, will be easier than larger throughput operations.
Laboratory false negatives	
Very low eDNA concentrations.	Samples with DNA amounts below the Limit of Detection will generate false negatives. It is not currently possible to mitigate this risk.

2.6 Laboratory specifications

2.6.1How the laboratory should be set up

The set-up of an eDNA laboratory should broadly follow the outline below. Note that this is not a detailed specification for building a laboratory: rather it provides guidance on the standard which is needed.

Successful eDNA work has so far been undertaken both in laboratories designed to standards established over the last 20 years for ancient DNA (aDNA) work (Knapp et al., 2012), and in more conventional DNA labs. There is as yet no evidence available to evaluate whether these different set-ups produce different results.

The main principles of the laboratory set-up should be (PHE, 2013):

- **Physical separation of pre and post-PCR work:** to prevent amplified DNA from contaminating samples there should be physical separation of pre and post PCR stages of the work. This should include separation of the area where sampling kits are prepared.
- **Unidirectional workflow**: The arrangement of activities in the rooms should be unidirectional to reduce potential for contamination. This can be achieved by physically having one room leading to another or by set working practices.

Two potential layouts of facilities based on existing constructed systems are exemplified below (Figure 1). The simpler design has some recognised limitations which are noted in the figure. Good results are known to have been produced in higher specification laboratories. The main features of the designs are:

- **Reagent preparation clean room:** a clean DNA free room is needed for the preparation of field sampling kits. Samples containing DNA should never be brought into this room and no DNA extractions or PCRs are performed in this room[1].
- **Nucleic acid extraction room**: the only area where DNA extractions are performed, and an area where PCR products and stocks of cloned material have not been handled.
- **Amplification room**: this is the area where PCR machines are housed.

The schematic designs shown in Figure 1 fulfil these criteria.

2.6.2Appropriate precautions to avoid laboratory contamination

As PCR products are ubiquitous in post-PCR laboratories it is important to make sure that no consumables or equipment for the DNA facility have been sourced from laboratories which undertake post-PCR amplification analysis.

Full body suits have been adopted by some eDNA laboratories for work in the sample kit preparation room and the eDNA sample preparation room, including full body suit, face masks, face shields and hairnets. In other rooms disposable laboratory coats are sufficient. Dedicated clean room shoes are useful to reduce carry-over contamination. Wearing two pairs of gloves will prevent exposure of skin when changing gloves. However, not all laboratory managers consider 'suiting-up' necessary, preferring separation of staff as the contamination control method (i.e. staff do not move between pre- and post-PCR laboratories). Those working with full body suits regard this approach as good practice for rare DNA work which generally reduces the amount of DNA present in the rooms to very low levels. Face masks reduce the breathing out of DNA which has been inhaled outside the clean rooms.

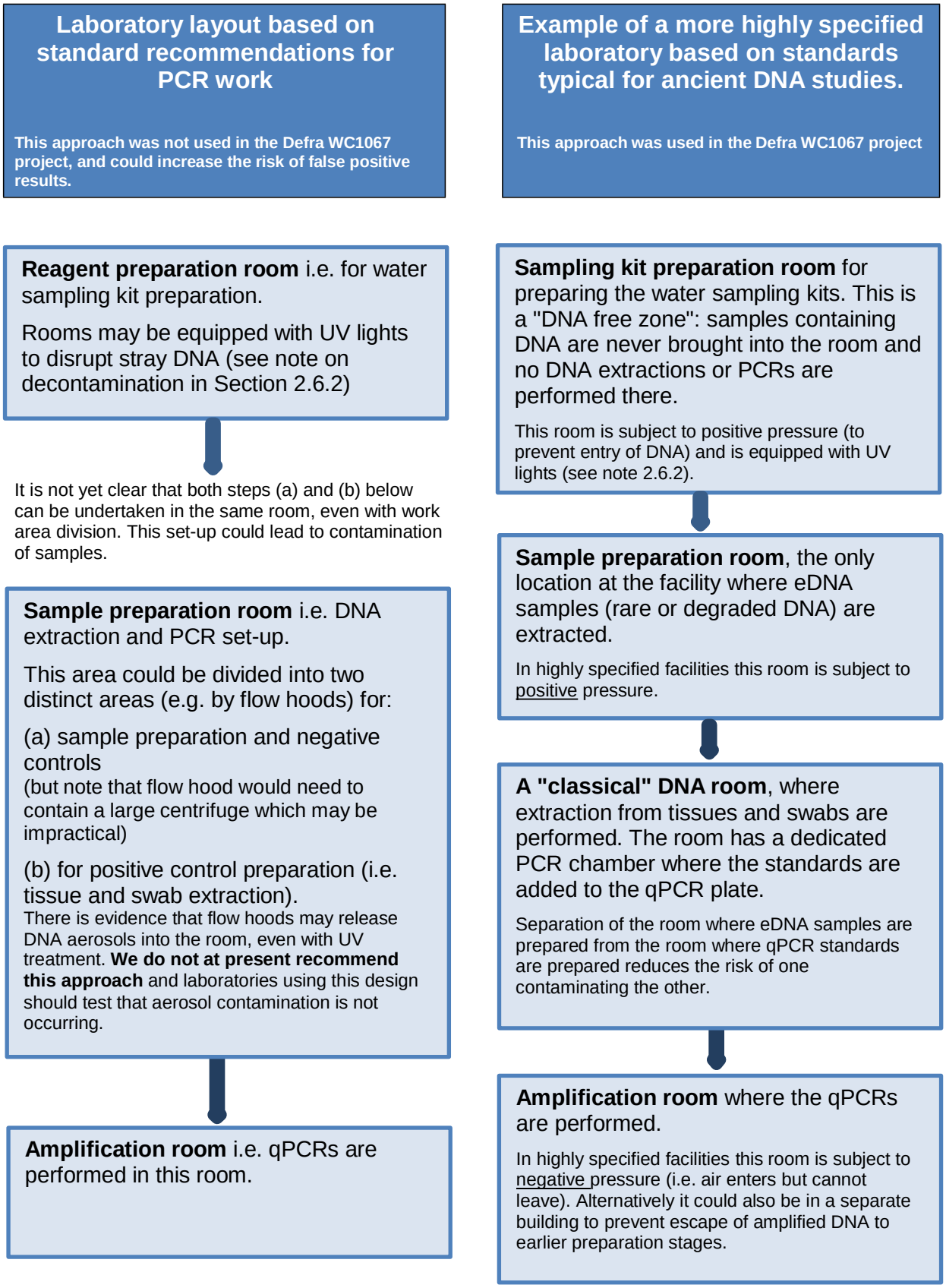
To reduce the risk of DNA contamination regular bleaching of the laboratory should be undertaken weekly. qPCR work should be undertaken inside a cabinet with UV light and in a room which is also lit by UV light outside the cabinet; to control aerosol DNA. Although UV lights are widely recommended for decontamination they need to be high power and close enough to the surface for decontamination to be effective and only then for low level contamination - cleaning and liquid decontamination is more effective (for detailed discussion see Champlot et al., 2010). They are also used in some laboratories to keep levels of environmental DNA low, including UV irradiating the facility when it is not in use.

Dedicated laminar flow hoods and fume hoods for DNA extraction and manipulation can reduce the risk of contamination still further. However, note that laminar flow hoods and fume hoods can under some circumstances make contamination worse by circulating contaminating aerosols around the laboratory. Most PCR hoods either do not have air flow, or are used switched off, providing a dedicated work station that is contained and can be easily decontaminated.

Further useful features are a positive pressure system and HEPA-filtered air conditioning. Some teams regard positive and negative pressure as desirable features to control contamination effectively. However, others suggest that procedural aspects are more important. At present it is not possible to tell which of these positions is correct. The more stringent standards of ancient DNA workers normally include positive / negative pressure and several successful laboratories working with eDNA have used this set-up. However, other groups have produced published results (e.g. Pilliod et al., 2013) without such systems. A highly specified laboratory (e.g. a forensic laboratory) may also have dedicated staff for each area because people are a major source of contamination. Vestibules with shoe/coat changing are effective techniques to prevent transfer of DNA in a highly specified laboratory, but can be adopted less expensively in less well specified laboratories by having dedicated shoes and coats for each laboratory.

[1] It is possible that a Class II cabinet in a non-DNA free room could be used for this step. If this approach is used it would be advisable for laboratories to demonstrate that this process did not lead to contamination of sample test kits. Cabinets are at risk of contamination from DNA aerosols which can be present in DNA laboratories even with UV lighting.

Figure 1. Examples of laboratory specifications proposed or in use for eDNA work.



3. Field protocol

Field sampling should be undertaken by a suitably trained and experienced great crested newt surveyor (trained volunteer or professional). At present it is believed that eDNA water sampling does not disturb newts enough to justify the procedure being licensed by the national regulatory authority.

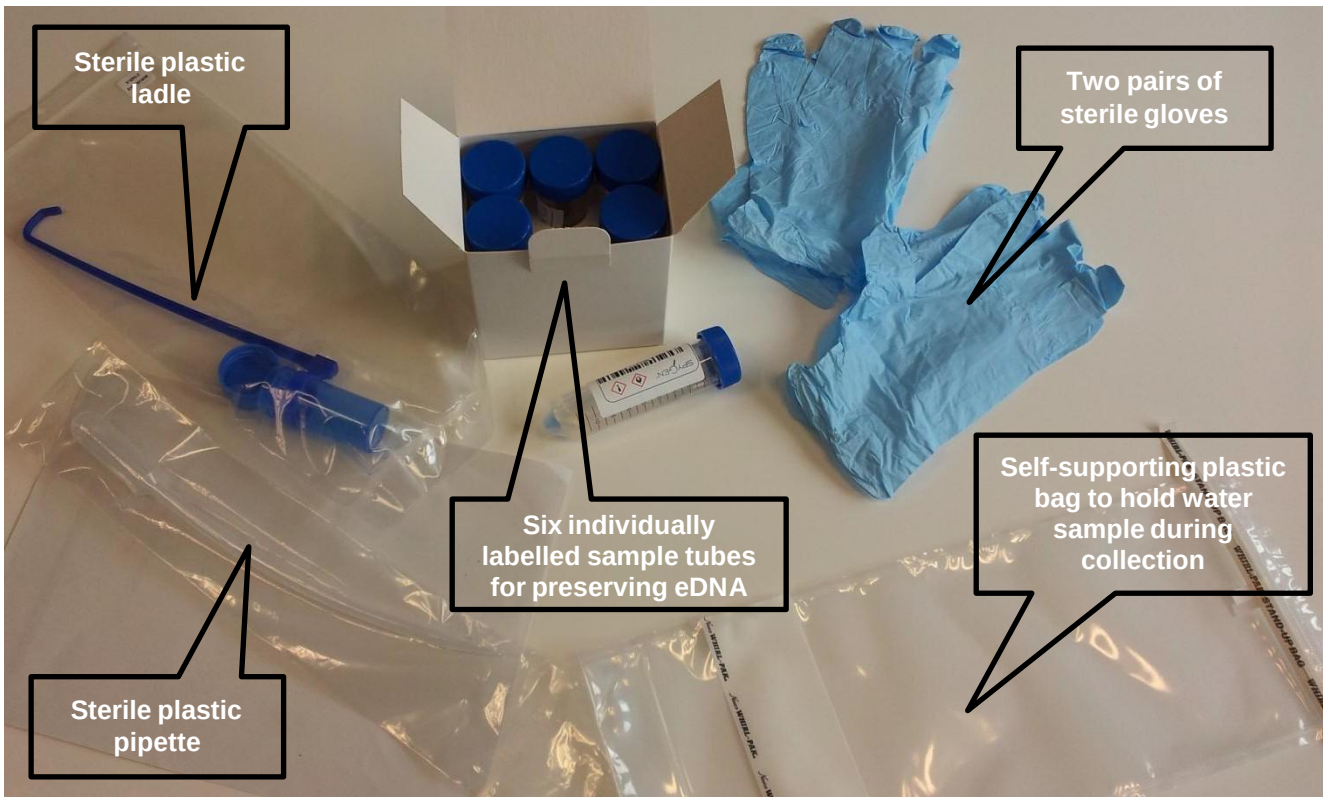
A single visit to the target pond should be made between mid-April and June, during the newt breeding season. eDNA samples can be collected at any time of day and in any reasonable weather conditions, including light rain. It may be best to avoid heavy rain as this makes sampling more difficult and might increase the risk of cross contamination (e.g. splashing of mud which could contain great crested newt DNA from wet ground). There is evidence that unpreserved amphibian eDNA decays slightly more quickly in full sun than shaded conditions, becoming undetectable after 8 and 11 days respectively (Pilliod et al., 2014), but as long as samples are preserved the impact on detection should be slight.

3.1 Sampling equipment

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

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

Figure 2 Sampling equipment used for eDNA water samples by Biggs et al. (2014)



Kits can be stored at room temperature before use in an appropriate solvent store, consistent with Home Office regulations, and should be used within about two weeks of receipt. The time between kit receipt and use should be noted (see Section 5.1). Use one kit per pond up to an area of 1 ha. Beyond this, use an additional kit per hectare. However, note that as yet there is no practical experience of the effectiveness of kits used on ponds greater than 1 ha in area. Note that sampling techniques are still developing rapidly in this field and alternative preservatives to ethanol are currently being sought.

3.2 Field water sample collection protocol

The field sampling protocol should follow the steps outlined below. Gloves should be worn at all times during the sampling process, replacing the gloves between sample collection from the pond and pipetting into the sterile sub-sample tubes. Samples should be collected without entering the water, i.e. the surveyor stands only on the pond bank or muddy pond edges. This prevents disturbance of the substrate and may limit cross-contamination.

Stages of field sampling protocol

Step 1 Identify where 20 samples will be taken from the pond. The location of sub-samples should be spaced as evenly as possible around the pond margin, and if possible targeted to areas where there is vegetation which may be being used as egg laying substrate and open water areas which newts may be using for displaying.

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

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

NOTE: Before each ladle sample is taken, the pond water column should be mixed by gently using the ladle to stir the water from the surface to close to the pond bottom without disturbing the sediment on the bed of the pond. It is advisable not to sample very shallow water (less than 5-10 cm deep).

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

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

Step 6 Using the clear plastic pipette provided take c15 mL of water from the Whirl-Pak bag and pipette into a sterile tube containing 35 mL of ethanol to preserve the eDNA sample (i.e. fill tube to the 50 mL mark). Close the tube ensuring the cap is tight.

Step 7 Shake the tube vigorously for 10 seconds to mix the sample and preservative. This is essential to prevent DNA degradation. Repeat for each of the 6 conical tubes in the kit. Before taking each sample, stir the water in the bag to homogenize the sample - this is because the DNA will constantly sink to the bottom.

Step 8 Empty the remaining water from the Whirl-Pack bag back into the pond.

Step 9 The box of preserved sub-samples is then returned at ambient temperature immediately for analysis. If batches of samples are collected and stored prior to analysis they should be refrigerated at 2-4° C. Kits can be stored for up to one month in a refrigerator before analysis. It is not necessary to freeze samples. Freezing may damage storage bottles, which can lead to leaking during transit, and also unnecessarily increases costs by requiring refrigerated transport. The length of time eDNA samples are stored in a refrigerator prior to analysis should be recorded and passed on to the analysing laboratory. Use an appropriate labelling system to ensure that the kits are supplied with a unique reference number.

4. Laboratory protocol

4.1 Introduction

This section describes the laboratory protocol for analysis of eDNA samples. It is assumed that laboratory staff are familiar with the techniques for using the proprietary products specified.

It is important that the analysing laboratory has no prior knowledge of whether sites being tested do or do not have newts. Samples should be identified only by a unique reference number which contains no site identifying information.

4.2 Analytical methods

Primers and probes

Great crested newt (*Triturus cristatus*) DNA should be amplified using the primers and probes listed in Appendix 2. They amplify a fragment of the mitochrondrial cytochrome oxidase I gene (cytb). It may be desirable for laboratories undertaking analyses to demonstrate that these primers and probes have been tested *in vitro* against real great crested newt tissue (which can be collected by external swabbing), and *in situ* from real ponds with great crested newts (unless they have already undertaken eDNA work with great crested newts). There are a number of amphibian biologists around the UK who have licenses to swab newts and they can be contracted to do this work. An alternative approach to standardisation is to purchase synthetic DNA.

Water

Water used in eDNA analysis should be ultrapure water for molecular biology grade, which can either be purchased or made in the laboratory, using proprietary equipment.

1. DNA extraction

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

Step 1 For each sample from a site, the six subsamples per site should be centrifuged at 14000 x g[1], for 30 minutes, at 6 °C and the supernatant discarded.

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

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

Step 4 An extraction control should be performed at the same time to monitor possible

[1]The centrifugation speed suggested originally by Ficetola et al. (2008) was 5500 x g. Internal tests made by Spygen indicated that better results were found with the highest centrifugation speed, which led to the adoption of 14,000 x g for the Great Crested Newt DNA extraction.

laboratory contamination. The extraction control is undertaken using an 11th tube containing buffers alone and no sample (i.e. no alcohol mix and no pond water). Note that the quality of the alcohol (i.e. absence of DNA contamination) is assessed with the negative controls in the field. These can be either out of range sites where great created newts are definitely absent or sites within the newt's range where there is high certainty that newts are absent. If no negative field sites are available in a study, a different approach may be needed. In the analytical process the extraction control sample is, from Step 4 onwards, processed as a normal sample.

Additional control samples may be added to the process depending on where it is believed contamination may be originating.

2. qPCR

Step 5 DNA inhibition should be tested by adding a known amount of an artificial gene tothe sample and running qPCR in duplicate. If a different than expected Ct[2] value is observed in a least one replicate, the sample should be considered inhibited. In this instance dilute the sample twice before amplification with great crested newt primer and probes.

Step 6 qPCR analysis. Each sample should be run in 12 replicates. A dilution series of *T. cristatus* DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-4} ng μL^{-1} (increments 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and measured using a Nanodrop ND-1000 or equivalent, should be used as a qPCR standard. The qPCR standards are made using DNA extracted from great crested newt tissue samples, and the quantification made on extracted DNA before the dilution. Samples should be run on a BIO-RAD® CFX96 Touch real time PCR detection system or equivalent.

Note that the standards are the positive controls for qPCR in this approach (i.e. assuring that the method successfully detects DNA when present). Negative controls are provided by one extraction blank, which is run with 12 replicates, as a normal sample, and with four qPCR negative controls which also run during the qPCR step, using ultrapure water for molecular biology grade.

Step 7 The quantitative PCR is performed in a final volume of 25 µL made up from:

- 3 µL of template DNA
- 12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®)
- 6.5 µL of ddH_2O
- 2 µL of primer (1 µL each of primer 10 µM TCCBL and TCCBR)
- 1 µL of probe (2.5 µM TCCB Probe)

Step 8 The PCR is performed under thermal cycling at 56.3 °C for 5 minutes and 95 °C for 10 minutes, followed by 55 cycles of 95 °C for 30 seconds and 52°C for one minute.

[2](C_t = C_t threshold value, the number of PCR cycles after which amplification becomes exponential)

5. Data recording and reporting

Accurate detailed records of the sites surveyed should be kept by the commissioning ecologists for reporting, reference and auditing purposes.

5.1 Sampling information

Sampling kits should be identified by a unique identifying code when provided to field ecologists. All site information should be associated with this unique number. Laboratory staff do not need further site reference information.

The commissioning ecologists should maintain records which include:

- Site name
- Nearest settlement (provides double check against grid reference errors)
- County (provides double check against grid reference errors)
- Time between receipt of sampling kit and date of sampling
- Date of sampling
- Personnel collecting sample
- Ordnance Survey grid reference, ideally to 1 m (i.e. a 12 figure grid reference)
- Site maps showing locations of sites
- Percentage of pond perimeter that is accessible for survey
- Data on inflows, and whether these were wet or dry at the time of survey
- If available, data on presence and number of great crested newt recorded during eDNA collection to help with further assessment / refinement of this technique
- Information on any difficulties experienced during sample collection.

5.2 Laboratory data

The laboratory should maintain records which include:

- Personnel involved identified
- Date of kit preparation
- Duration of storage of samples once returned from the field
- Dates of analysis
- Details on type and any degradation of the marker DNA in sample kits
- A record of any modifications to standard operation procedures of laboratory equipment.

Standard laboratory data should be maintained by the laboratory.

Information on sample inhibition should be reported with the reporting of positive or negative DNA results.

At present there is no intention to archive eDNA samples although this may become necessary in the future.

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Appendix 1. Frequently asked questions about eDNA sampling from volunteer and non-specialist surveyors

What is eDNA?
eDNA in the case of larger organisms, is DNA that is collected from the environment in which an organism lives, rather than directly from the plants or animals themselves. 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 in the waterbody.

Why must surveyors remain out of the pond?
There is a considerable risk of contaminating your pond sample by bringing in Great Crested Newt DNA in mud and water from other areas on your boots and equipment. This is a real risk: DNA can remain on surfaces even after they have been dried, and can persist in soil for many years. There are recorded examples of eDNA cross-contaminating pond water samples from surveyor's boots.

Why are sampling points spread around the pond?
Existing data shows that eDNA can be very patchy depending on where the animals have been. By sampling in many areas you considerably increase your chance of collecting their DNA successfully.

Why is the water column mixed before sampling?
DNA 'sinks' and so will often be present in larger amounts close to the pond bottom. However, it is important not to collect sediment because it is currently thought that DNA may persist in the sediment for substantially longer than in the water column. If you collect sediment, there is a risk your sample might show a false positive indicating great crested newts were present recently, when in fact this was a long time in the past.

Why is such a large volume of water collected?
In this methodology we collect a larger volume of water than previous methods have recommended (e.g. Thomsen et al. 2012). Our experience indicates that collecting a larger volume of water than was taken by Thomsen et al. (2012) is important to the success of the method.

Does it matter if I get things like duckweed, algae or zooplankton in my sample?
No, small amounts don't matter. However try not to collect bottom sediment in the sample, because the DNA can be absorbed by sediment and may give false positive results (see above).

What happens if I spill the preservative - or the sample tube itself
If you spill some of the preservative from one of the tubes, just add proportionally less water from your pond sample. The samples from all six tubes are later combined for the laboratory analysis, so it's not disastrous if some sample is lost.

Will samples degrade in the post?
The preservative (alcohol) in the sample bottle will slow, but not eliminate, degradation of any DNA. Keeping the samples refrigerated also slows this process. The rate of decay during posting at ambient temperatures will be faster, but it will not be sufficient to degrade the sample completely.

What evidence is there to support the use of this technique?
Defra project WC1067 has demonstrated the effectiveness of environmental DNA in the detection of Great Crested Newts. In detailed field studies eDNA detected Great Crested Newts 99.3% of the time in ponds where they were known to occur. When used by volunteers 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.

Appendix 2 Details of primers and probes

Primers are salt free and HPLC-purified.

Primer	Sequence (5'-'3)	Fragment	Gene
TCCBL	CGTAAACTACGGCTGACTAGTACGAA	81	Cyt-b
TCCBR	CCGATGTGTATGTAGATGCAAACA	81	Cyt-b
Probe			
TCCB	CATCCACGCTAACGGAGCCTCGC	81	Cyt-b

Degradation control

A length of artificial DNA is added to the samples to assess post-sampling degradation. This DNA does not have an analogue in the natural world so it can clearly be separated from all DNAs that can be sampled in the field. The structure of the molecule is commercially confidential to Spygen so is not reproduced in this guide. Laboratories may either design their own synthetic DNA or purchase material from Spygen.

Acknowledgements

We would like to thank all those who helped with Defra project WC1067 including the landowners who facilitated access to their sites, and particularly the many people and groups who volunteered time and resources to collect eDNA samples. This includes NARRS and PondNet volunteers, the team in Wales co-ordinated by Matt Ellis of Natural Resources Wales, who generated an excellent dataset for the detailed methodological component of the project, and Tom Langton who not only collected many eDNA samples, but provided access to a dataset from Suffolk extending over 20 years which has helped us to better interpret the relationship between eDNA and Great Crested Newt abundance. The project lead was Natasha Chick (Defra) and the Steering Group was Matt Ashton (Defra), Pete Brotherton (Natural England), Paul Edgar (Natural England), John McKinnel (Scottish Natural Heritage), Katharine Woods (Natural England) and Anna Robinson (JNCC). Our thanks also to Barbara Zweifel for delivering eDNA samples to France for analysis.

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Appendix I: Technical Reports - GCN eDNA Analysis (SureScreen Scientifics)

Folio No: 2534-2025
Purchase Order: DG00073
Contact: Dawn Goodfellow
Issue Date: 01.07.2025
Received Date: 17.06.2025

GCN Report

Technical Report

SureScreen Scientifics

Folio No: 2534-2025
Purchase Order: DG00073
Contact: Dawn Goodfellow
Issue Date: 01.07.2025
Received Date: 17.06.2025

SureScreen Scientifics

GCN eDNA Analysis

Summary

When great crested newts (GCN), *Triturus cristatus* , inhabit a pond, they continuously release small amounts of their DNA into the environment. By collecting and analyzing water samples, we can detect these small traces of environmental DNA (eDNA) to confirm GCN habitation or establish GCN absence.

Results

Lab ID	Site Name	OS Reference	Degradation Check	Inhibition Check	Result	Positive Replicates
R25 1092	Secret Lake - P1	SJ 88735 94620	Pass	Pass	Negative	0/12

Matters affecting result: none

Reported by: Amy Bermudez

Approved by: Consuela Sopronyi

SureScreen Scientifics

Methodology

The samples detailed above have been analyzed for the presence of GCN eDNA following the protocol stated in DEFRA WC1067 'Analytical and methodological development for improved surveillance of the Great Crested Newt, Appendix 5.' (Biggs et al. 2014). Each of the 6 sub-sample tubes are first centrifuged and pooled together into a single sample tube which then undergoes DNA extraction. The extracted sample is then analyzed using real-time PCR (qPCR), which uses species-specific molecular markers to amplify GCN DNA within a sample. These markers are unique to GCN DNA, meaning that there should be no detection of closely related species.

If GCN DNA is present, the DNA is amplified up to a detectable level, resulting in positive species detection. If GCN DNA is not present then amplification does not occur, and a negative result is recorded. Analysis of eDNA requires attention to detail to prevent the risk of contamination. True positive controls, negative controls, and spiked synthetic DNA are included in every analysis and these have to be correct before any result is declared and reported. Stages of the DNA analysis are also conducted in different buildings at our premises for added analytical security.

SureScreen Scientifics Ltd is ISO9001 accredited and participates in Natural England's proficiency testing scheme for GCN eDNA testing.

Interpretation of Results

Sample Integrity Check: When samples are received in the laboratory, they are inspected for any tube leakage, suitability of sample (not too much mud or weed etc.) and absence of any factors that could potentially lead to inconclusive results. Any samples which fail this test are rejected and eliminated before analysis.

Degradation Check: **Pass/Fail**. Analysis of the spiked DNA marker to see if there has been degradation of the kit or sample between the date it was made to the date of analysis. Degradation of the spiked DNA marker may lead indicate a risk of false negative results.

Inhibition Check: **Pass/Fail.** The presence of inhibitors within a sample is assessed using a DNA marker. If inhibition is detected, samples are purified and re-analyzed. Inhibitors cannot always be removed, if the inhibition check fails, the sample should be re-collected.

Result: **Presence of GCN eDNA (Positive/Negative/Inconclusive)**
Positive: GCN DNA was identified within the sample, indicative of GCN presence within the sampling location at the time the sample was taken or within the recent past at the sampling location.
Positive Replicates: Number of positive qPCR replicates out of a series of 12. If one or more of these are found to be positive the pond is declared positive for GCN presence. 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 the WC1067 Natural England protocol, even a score of 1/12 is declared positive. 0/12 indicates negative GCN presence.
Negative: GCN eDNA was not detected or is below the threshold detection level and the test result should be considered as evidence of GCN absence, however, does not exclude the potential for GCN presence below the limit of detection.
Inconclusive: Controls indicate inhibition or degradation of the sample, resulting in the inability to provide conclusive evidence for GCN presence or absence.

Folio No: 2535-2025
Purchase Order: DG00074
Contact: Dawn Goodfellow
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GCN Report

Technical Report

SureScreen Scientifics

Folio No: 2535-2025
Purchase Order: DG00074
Contact: Dawn Goodfellow
Issue Date: 01.07.2025
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SureScreen Scientifics

GCN eDNA Analysis

Summary

When great crested newts (GCN), *Triturus cristatus* , inhabit a pond, they continuously release small amounts of their DNA into the environment. By collecting and analyzing water samples, we can detect these small traces of environmental DNA (eDNA) to confirm GCN habitation or establish GCN absence.

Results

Lab ID	Site Name	OS Reference	Degradation Check	Inhibition Check	Result	Positive Replicates
R25 1093	Secret Lake - P2	SJ 89039 94711	Pass	Pass	Negative	0/12

Matters affecting result: none

Reported by: Amy Bermudez

Approved by: Consuela Sopronyi

SureScreen Scientifics

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