

Land to the North of The Elms, Darland Lane,
Rossett - Great Crested Newt Survey (June 2018)

Carried out by Amenity Tree Care

23 07 18

Version 1

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1.0 EXECUTIVE SUMMARY

- 1.1 Amenity Tree Care Ltd. (Amenity) was commissioned to undertake a great crested newt (GCN) aquatic survey and terrestrial appraisal to assess the predicted development effect and formulate appropriate and proportionate GCN/amphibian mitigation and habitat enhancement measures in connection with proposed works on land near Darland Lane, Rossett, North Wales. However, the exact proposals for the site are unknown.
- 1.2 The total site area extends to approximately 0.9 hectares. The site is located within a rural setting on the north-east edge of the village of Darland, near Rossett with residential areas to the west surrounding the site entrance and agricultural land surrounding the site on all other sides. The wider area comprised arable land, woodland and ponds.
- 1.3 At the time of the survey terrestrial habitat at the site comprised a flat concrete apron with an existing metal building present with associated parking/storage areas, a rubble pile, surrounding tall ruderal herbs and an area of amenity grass.
- 1.4 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.
- 1.5 The review of OS and aerial mapping / imaging, and a walk-over inspection of the area of works and neighbouring land by Amenity ecologists on 11th June 2018, established that four water bodies (Water bodies 1 - 4) were extant either on or within approximately 250 metres of the area of works.
- 1.6 The review of OS and aerial mapping / imaging and the walk-over inspection established that a single further water body, (Water body A), was also extant at a distance of approximately 250 metres up to 500 metres from the area of works, though it was noted that this brook connects with several ditch systems.
- 1.7 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 Water body 1 was relevant to the survey. Water body 1 was visited and photographed, a HSI calculated and a GCN eDNA sample was obtained and later analysed.

- 1.8 However, Water bodies 2 - 4 & A were not considered relevant and were therefore excluded from the survey on the grounds that they were effectively isolated from the area of works by distance and/or barriers to dispersal, i.e. roads and/or built environments and/or restricted habitat connectivity, and/or did not offer GCN suitable aquatic breeding habitat.
- 1.9 The methods employed included a combination of visual inspection and GCN eDNA water sampling, as appropriate to timing of the survey and the conditions encountered. As part of the survey a GCN 'Habitat Suitability Index' (HSI) score and categorisation was calculated for Water body 1.
- 1.10 The HSI score calculated for Water body 1 indicated 'excellent' habitat suitability for GCN.
- 1.11 Analysis of the sample collected for GCN eDNA later robustly established GCN presence at Water body 1 at the current time.
- 1.12 At the time of the survey terrestrial habitat to be directly impacted by the area of works was assessed to offer GCN and other amphibians with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Below Average'.
- 1.13 Consequently, it has been concluded that GCN were reasonably unlikely to be present within the area/s that will be directly affected by the works.
- 1.14 No GCN breeding sites were adversely impacted as a consequence of the works.
- 1.15 The works would be likely to result in; partial destruction, temporary disturbance and/or temporary destruction followed by reinstatement and modified management of a relatively small area of intermediate (>50 metres to <250

metres) and distant (>250 metres) terrestrial habitat associated with Water body 1 and/or other ponds/water bodies in the local area (defined by Natural England as within 10 kilometres).

- 1.16 The works will not impact negatively on habitat connectivity and / or the ability of GCN/amphibians to migrate and disperse.
- 1.17 The works will not significantly increase (from current and historic levels) the risk of post-development human interference impacts on any GCN / amphibian population/s and / or aquatic and terrestrial habitats associated with the local area.
- 1.18 The predicted development effect and scale of impact has been assessed, with reference to the English Nature Great Crested Newt Mitigation Guidelines 2001 and, in the absence of appropriate mitigation measures, a 'low' negative development impact was predicted for any GCN / amphibian populations potentially associated with Water body 1 and/or the local area.
- 1.19 It has been concluded that the unmitigated commencement of development at the site could potentially have adverse impacts on individual GCN and/or other amphibians in the unlikely event that they are present or inadvertently stray into the working area following the commencement of development at the site and take cover under/within temporary shelter habitat incidentally created as a consequence of the works, i.e. excavations, spoil mounds and/or stored materials.
- 1.20 Following careful consideration of the survey findings and given the limited/localised scale of the development, the predicted 'low' negative development impacts, the legal protection afforded to the species and current guidance issued by Natural England in respect of low impact development projects, it was deemed appropriate to recommend a GCN mitigation strategy involving the implementation of non-licensed Reasonable Avoidance Measures (RAM) and habitat enhancement measures in respect of the proposed development.

- 1.21 Amenity have concluded that the RAM and habitat enhancement measures proposed and detailed in this report are in accordance with current Natural England guidance and that their successful implementation will effectively minimise the risk of breaching current legislation relating to GCN and will ensure that the favourable conservation status of the GCN population/s identified to be associated with Water body 1 is maintained within their natural range.

2.0 INTRODUCTION

- 2.1 Amenity Tree Care Ltd. (Amenity) was commissioned to undertake a great crested newt (GCN) aquatic survey and terrestrial appraisal to assess the predicted development effect and formulate appropriate and proportionate GCN/amphibian mitigation and habitat enhancement measures in connection with proposed works on land near Darland Lane, Rossett, North Wales. However, the exact proposals for the site are unknown.
- 2.2 The Amenity 2018 survey was led by Dawn Goodfellow, BSc (Hons) MCIEEM (Senior Ecologist). Dawn holds a Natural Resources Wales GCN survey licence (No. 75822:OTH:SA:2017) 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 been trained in how to take 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;
- 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 total site area extends to approximately 0.9 hectares. The site is located within a rural setting on the north-east edge of the village of Darland, near Rossett with residential areas to the west surrounding the site entrance and agricultural land surrounding the site on all other sides. The wider area comprised arable land, woodland and ponds (see Appendix D: Site Location Plan (OS) and Appendix E: Photographic Plates).

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

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

Habitat type	Approximate Area (Ha)/length (m)	Suitability for GCN/Amphibians
Amenity Grassland	0.7925	'Below Average'
Building	0.013	'Poor'
Compacted ground (track)	0.0152	'Poor'
Concrete apron	0.027	'Poor'
Intact species-poor hedgerow	59m	'Average'

Intact species-rich hedgerow	110m	'Above Average'
Scattered trees	0.0095	'Average'
Tall ruderal herbs	0.0428	'Average'

- 3.3 At the time of the survey terrestrial habitat to be directly impacted by the area of works was assessed to offer GCN and other amphibians with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Below Average'.
- 3.4 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).
- 3.5 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.
- 3.6 At the time of the survey terrestrial habitat at the site comprised a flat concrete apron with an existing metal building present with associated parking/storage areas, a rubble pile, surrounding tall ruderal herbs and an area of amenity grass.
- 3.7 The review of OS and aerial mapping / imaging, and a walk-over inspection of the area of works and neighbouring land by Amenity ecologists on 11th June 2018, established that four water bodies (Water bodies 1 - 4) were extant either on or within approximately 250 metres of the area of works.
- 3.8 The review of OS and aerial mapping / imaging and the walk-over inspection established that a single further water body, (Water body A), was also extant at

a distance of approximately 250 metres up to 500 metres from the area of works, though it was noted that this brook connects with several ditch systems.

- 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 Water body 1 was relevant to the survey. Water body 1 was visited and photographed, a HSI calculated and a GCN eDNA sample was obtained and later analysed.
- 3.10 However, Water bodies 2 - 4 & A were not considered relevant and were therefore excluded from the survey on the grounds that they were effectively isolated from the area of works by distance and/or barriers to dispersal, i.e. roads and/or built environments and/or restricted habitat connectivity, and/or did not offer GCN suitable aquatic breeding habitat.
- 3.11 Additional information relating to the water bodies excluded from the survey is detailed below in Table 2.

Table 2: Water bodies excluded from survey

Water body	Distance from proposed development site boundary (m)	Location/setting	Barriers/constraints to GCN/amphibian dispersal	Other reason/s for exclusion
2	25m	Garden pond	-	Likely presence of fish so considered unsuitable for GCN/amphibian breeding.
3	210m	Field pond to the SE	Distance from the site and the presence of barriers to dispersal including commercial areas. Lack of habitat connectivity.	-
4	230m	Woodland pond to the W	Distance from the site and the presence of barriers to dispersal including roads and residential areas. Lack of habitat connectivity.	-
A	400m	Pulford Brook (ditch to the N)	Distance from the site and the presence of barriers to dispersal (including arable land). Lack of habitat connectivity.	A flowing watercourse considered unsuitable for GCN/amphibian breeding.

N.B. - Distance and area measurements taken from available Ordnance Survey and Aerial mapping.

- 3.12 A brief description of the aquatic habitat at Water body 1 (included in the survey) has been provided for clarity (refer to Appendix E: Photographic Plates).

Water body 1: Grid Ref: SJ 38010 58130

This pond was holding water at the time of survey and had a moderate diversity of aquatic vegetation, although there was little open water present. Water quality was considered to be moderate and it was considered never likely to dry out during years of drought. The pond was bounded by trees around approximately two-thirds of its perimeter and set within an arable landscape. Only minor impacts were observed from wildfowl presence. No fish were recorded during the course of the survey, although fish presence was considered possible, though unlikely.

- 3.13 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.
- 3.14 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.

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 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 result. Samples must be analysed using a strict eDNA screening method issued by Natural England (WC1027), (refer to Appendix H: Natural 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 2018 GCN survey was led by Dawn Goodfellow BSc (Hons) MCIEEM (Senior Ecologist). The survey effort deemed appropriate in respect of the proposed development 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 Water body 1.
- 4.14 The survey was undertaken in accordance with the terms and conditions of a Natural Resources Wales GCN Survey Licence (No. 75822:OTH:SA:2017). The survey effort included a review of OS and aerial mapping/imaging and a single, daytime visit/walk-over inspection of the site, by Amenity ecologists on the 11th June 2018, in order to assess the status of habitats at Water body 1 and at the proposed development site.

- 4.15 The methods employed at Water body 1 included GCN eDNA water sampling and analysis for GCN presence/absence, as appropriate to the timing of the visit and the conditions encountered.
- 4.16 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 North West 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 North West 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, as a consequence of the widespread status of the species within the North West 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.
- 5.4 The main findings of the Amenity 2018 GCN survey undertaken in relation to the area of works are detailed below:
- 5.5 The HSI score calculated for Water body 1 indicated 'excellent' suitability for GCN.
- 5.6 The survey robustly established GCN presence at Water body 1 at the current time.

- 5.7 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 limited shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Below Average'.
- 5.8 Consequently, it has been concluded that GCN were reasonably unlikely to be present within the area/s that will be directly affected by the works.
- 5.9 The GCN 'Habitat Suitability Index' scores/categorisation and GCN eDNA survey result for Water body 1, is detailed in Tables 3 & 4 below.

Table 3: HSI score/categorisation (Water body 1)

	Water body 1
Pond Ref:	
SI1 - Location	1
SI2 - Pond area	0.85
SI3 - Pond drying	0.9
SI4 - Water quality	0.67
SI4 - Shade	1
SI6 - Fowl	0.67
SI7 - Fish	0.67
SI8 - Ponds	1
SI9 - Terr'l habitat	0.67
SI10 - Macrophytes	0.85
HSI	0.82
Categorisation	Excellent

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

Table 4 (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							
Date Sample Received at Laboratory:	12/06/2018						
Date Reported:	20/06/2018						
Matters Affecting Results:	None						
<u>Results:</u>							
Lab Sample No.:	Site Name:	OS Ref:	SIC:	DC:	IC:	Result:	Positive Replicates:
1801	Water body 1	SJ 38010 58130	Pass	Pass	Pass	Positive	12

- 5.10 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 No GCN breeding sites will be adversely impacted as a consequence of the works.
- 6.2 The works would be likely to result in; partial destruction, temporary disturbance and/or temporary destruction followed by reinstatement and modified management of a relatively small area of intermediate (>50 metres to <250 metres) and distant (>250 metres) terrestrial habitat associated with Water body 1 and/or other ponds/water bodies in the local area (defined by Natural England as within 10 kilometres).
- 6.3 The works will not impact negatively on habitat connectivity and / or the ability of GCN/amphibians to migrate and disperse.

- 6.4 The works will not significantly increase (from current and historic levels) the risk of post-development human interference impacts on any GCN / amphibian population/s and / or aquatic and terrestrial habitats associated with the local area.
- 6.5 The predicted development effect and scale of impact has been assessed, with reference to the English Nature Great Crested Newt Mitigation Guidelines 2001 and, in the absence of appropriate mitigation measures, a 'low' negative development impact was predicted for any GCN / amphibian populations potentially associated with Water body 1 and/or the local area.
- 6.6 The predicted development effect and impacts are detailed/highlighted (blue) in Table 5 below (adapted from the EN GCN MG).

Table 5: 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 likely to be used by the species)	Destruction			✓
	Isolation caused by fragmentation			✓
	Partial destruction		✓	
	Modified management, resurfacing etc.		✓	
	Temporary disturbance	✓		
	Post-development interference		✓	
	Temporary destruction & reinstatement	✓		
Intermediate Terrestrial Habitat: (at a distance of 50 metres up to 250 metres from a GCN/breeding pond or other water body likely to be used by the species))	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 by the species)	Destruction	✓		
	Isolation caused by fragmentation	✓		
	Partial destruction	✓		
	Modified management, resurfacing etc.	✓		
	Temporary disturbance	✓		
	Post-development interference	✓		
	Temporary destruction & reinstatement	✓		

- 6.7 It has been concluded that the unmitigated commencement of development at the site could potentially have adverse impacts on individual GCN and/or other amphibians in the unlikely event that they are present or inadvertently stray into the working area following the commencement of development at the site and take cover under/within temporary shelter habitat incidentally created as a consequence of the works, i.e. excavations, spoil mounds and/or stored materials.
- 6.8 Following careful consideration of the survey findings and given the limited/localised scale of the development, the predicted 'low' negative development impacts, the legal protection afforded to the species and current guidance issued by Natural England in respect of low impact development projects, it was deemed appropriate to recommend a GCN mitigation strategy involving the implementation of non-licensed Reasonable Avoidance Measures (RAM) and habitat enhancement measures in respect of the proposed development.

7.0 RECOMMENDED REASONABLE AVOIDANCE MEASURES & HABITAT ENHANCEMENT STRATEGY

- 7.1 Amenity have concluded that the RAM and habitat enhancement measures proposed and detailed below are in accordance with current Natural England guidance and that their successful implementation will effectively minimise the risk of breaching current legislation relating to GCN and will ensure that the favourable conservation status of the GCN population/s identified to be associated with Water body 1 is maintained within their natural range.
- 7.2 Current Natural England GCN guidance states:
"In recent years there has been a trend towards increasingly precautionary licence applications, resulting from a risk-averse approach to mitigation. Whilst considering potential risks to great crested newts is laudable, many recent mitigation schemes were designed for developments that actually had very little or no effect on the newt population. In part this is because it can be difficult to assess whether newts will be affected by certain activities, especially when they take place at some distance from breeding ponds. Newts tend to be present at increasingly low density the further one looks from ponds, and the task of

detecting and capturing them becomes more problematic. Further from ponds, there is a corresponding reduction in the scale of impact on populations. Given that great crested newts can disperse over 1km from breeding ponds, the potential for offences may seem vast, yet the probability of an offence outside the core breeding and resting area is often rather small, and even if an offence takes place, the effect on the population may be negligible'.

'Natural England is concerned about the trend for increasingly risk-averse mitigation for several reasons. Primarily, there is no legal need, and little benefit to great crested newt conservation, in undertaking mitigation where there are no offences through development. Even where there technically is an offence, such as the destruction of a small, distant area of resting place habitat, or even killing low numbers of newts, it is arguable that impacts beyond the core area often have little or no tangible impact on the viability of populations. Mitigation in such circumstances is of questionable value in conservation terms. There are, however, substantial costs: developers delay projects and spend large sums on mitigation. Sometimes the mitigation project itself has environmental costs, especially when it entails substantial lengths of newt fencing. In some cases long newt fences are employed with no justification. Natural England wishes to see newt fencing used more appropriately, i.e. only where there is a reasonable risk of capturing, containing and/or excluding newts'.

'Natural England recognises that the two key factors leading consultants to adopt this risk-averse approach are: (a) uncertainty over the presence of newts and whether there will be an offence in areas distant from ponds; (b) undertaking mitigation under licence "just in case", so that there is no perceived risk of litigation for their client. Natural England wishes to see mitigation planning shift away from such a highly risk-averse starting point. The domestic legislation protecting great crested newts arises largely from the Habitats Directive, which has a central aim to restore scheduled species to a favourable conservation status. A more proportionate approach to mitigation, addressing tangible impacts on populations whilst giving lower priority to negligible effects, is consistent with the aims of the Directive. It remains the responsibility of the developer - normally acting through their ecological consultant - to decide whether to apply. Early consideration of options including the implementation

of reasonable avoidance measures can often result in no licence being required”.

Reasonable Avoidance Measures to be Implemented:

1. Prior to commencing development at the site a suitably experienced and GCN licensed consultant ecologist should be appointed by the developer to ensure that the RAM are successfully implemented.
2. The RAM will be implemented as deemed appropriate by the appointed ecologist. A copy of this report and supporting materials including GCN/amphibian identification sheets and 'on call' ecologist contact details should be kept on-site and available for inspection during development related works at the site.
3. Prior to any development related works commencing at the site the developer/contractors should undergo a site induction/tool box talk where the implications arising from the presence of GCN/amphibians within the local area and the need to implement the RAM detailed in this report should be fully explained by the appointed ecologist.
4. The timing of the development works and implementation of RAM will, where practicable to do so, encompass daylight hours and the period when the majority of GCN/amphibians are considered likely to be at their breeding sites and/or not active above ground. Consequently the risk of GCN/amphibians being adversely impacted by the works will be minimised.
5. Prior to any vegetation clearance and/or ground disturbance works commencing at the site, the extent of works should be agreed by the appointed ecologist and the developer/contractors.
6. All trees and hedgerows that are required to be retained at or neighbouring the development site should be protected from direct impact and severance or asphyxiation of root systems. Any ground disturbance works should only take place at a minimum distance from individual trees of branch spread or half-tree height, (whichever is the greater).
7. Any required tree and hedgerow protection measures should be implemented in accordance with current best practice recommendations and guidance.
8. Terrestrial habitat protection measures, including the use of exclusion zones, should be implemented as deemed appropriate. The appointed ecologist and the developer/contractors should identify, agree and appropriately secure any required terrestrial habitat exclusion zones prior to the commencement of development works at the site.

9. Vegetation removal/management may be used to discourage GCN/amphibians from entering areas of the development site that are to be subject to significant ground disturbance works.
10. Where vegetation removal/management works, with the potential to disturb nesting birds, including the required removal of trees and/or hedgerows at the development site, cannot be scheduled outside of the bird nesting season (March to September) the appointed ecologist or their accredited agent should carry out a nesting bird survey at, and neighbouring, the proposed area of works.
11. A nesting bird survey should be undertaken immediately prior to the commencement of any significant vegetation/tree/shrub/bramble removal works at the development site between March and September.
12. In the event that nesting birds are found to be present at the development site, it will be necessary to formulate and implement a mitigation strategy (dependent on the bird species) to address their presence and prevent any disturbance to the nest site and a breach of wildlife legislation.
13. To minimise the risk to GCN/amphibians during vegetation removal/management works hand-held trimmers and/or brush-cutters or low ground impact machinery i.e. quad and flail should be used. This will prevent ground compaction and any inadvertent damage to the areas of terrestrial habitat/vegetation at and adjacent the development site that is to be retained.
14. In accordance with best practice recommendations all vegetation removal works should ideally be undertaken in stages and under the supervision of the appointed ecologist or their accredited agent to limit disturbance and allow animals to safely vacate the area.
15. The appointed ecologist and the developer/contractors should ensure that no areas of aquatic or terrestrial habitat that are to be retained at/adjacent/neighbouring the development site are inadvertently lost or damaged as a consequence of the development works.
16. A search of potential places of refuge within the development site should be carried out (where practicable to do so) by the appointed ecologist or their accredited agent immediately prior to the commencement of any significant ground disturbance works at the site in order to check for sheltering GCN/amphibians. The search must be undertaken under the terms/conditions of a current GCN class licence.
17. As deemed appropriate by the appointed ecologist, items of potential refuge should be removed from the development site immediately following the search to prevent GCN/amphibians from seeking shelter either beneath or within them.

18. The development site, together with any on-site storage/lay down areas, should be kept clear of debris and, where practicable; stored materials should be kept off the ground on stillages or pallets so as to prevent GCN/amphibians from seeking shelter or protection under/within them.
19. Where materials need to be delivered to the development site for immediate use and/or temporary storage, directly on the ground, care should be taken not to cause unnecessary or inadvertent damage or disturbance to neighbouring terrestrial habitat.
20. Any skips or bins, if used, should ideally be stored on baulks of timber to keep them off the ground so as to prevent GCN/amphibians from seeking shelter under them.
21. In the event that spoil needs to be removed from the development site, it should be taken off-site at the earliest opportunity for appropriate disposal.
22. The appointed ecologist should advise the developer/contractor to consider potential bio-security issues relating to the import and removal of material at the development site and the need to undertake a bio-security risk assessment and/or implement measures to prevent the inadvertent spread of non-native species, disease and biological pathogens.
23. Should any excavation/s be left open overnight for any reason, the excavation/s should be searched and checked for sheltering GCN/amphibians. The search should be carried out by the appointed ecologist or their accredited agent before works re-commence.
24. All open excavations should ideally incorporate soil 'ramps' at either end to allow GCN/amphibians and small mammals falling into them to escape.
25. Wherever practicable, excavations should be in-filled and made good to ground level at the earliest opportunity, so as to remove any hazard to GCN/amphibians.
26. Should spoil/materials be left on the ground overnight they may require searching for sheltering GCN/amphibians by the appointed ecologist or their accredited agent before they are moved.
27. Temporary Amphibian Fencing (TAF) may be used at the development site in order to discourage GCN/amphibians from entering the working area and taking shelter within open excavations, disturbed ground, spoil mounds created as a consequence of the development works.
28. In accordance with current Natural England and best practice guidance, the use of TAF (upright and/or one-way) at the development site will be at the

discretion of the appointed ecologist and for a limited period until the works are complete.

29. TAF should be used as a short term measure only if it is considered likely to reduce the risk of injury to GCN/amphibians and where its use would have no significant impact on their ability to migrate/disperse and reach their breeding/shelter sites. The use of one-way TAF will allow amphibians and/or other wildlife i.e. small mammals to safely vacate the site into the wider area and will prevent them re-entering the working area
30. TAF installation, inspections, maintenance and removal should be carried out by the appointed ecologist or their accredited agent in accordance with current Natural England and best practice guidance.
31. In respect of the proposed development, the short term installation of TAF (upright and/or one-way) at the development site would, in the view of Amenity, not result in substantial interference to the dispersal routes of GCN/amphibians and would be unlikely to result in a breach of current legislation relating to GCN.
32. If used, the TAF should be inspected on a regular basis and maintained in good order. A detailed record should be kept of the TAF installation date, rationale for its use, the dates of the inspection visits and any repairs undertaken.
33. Should amphibians (other than GCN) be found terrestrially at the development site they will be captured by hand and may be held captive for a short period in secure, clean container/s lined with damp moss prior to their release into suitable cover adjacent the site boundary, as deemed appropriate by the appointed ecologist.
34. 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. Amphibian capture should be carried out by the appointed ecologist or their accredited agent.
35. In the event that ecological supervision is not present on-site when an amphibian is found terrestrially the developer/contractor should place the amphibian/s into the secure, clean container/s lined with damp moss provided by the appointed ecologist and to be kept on-site for such an eventuality. The appointed ecologist or their accredited agent should be contacted immediately (using the 'on call' service, if appropriate) for advice on release and to ensure that the amphibian species is correctly identified/recorded.
36. The winter hibernation/dormancy period for amphibians is generally accepted to run from November to February, 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 amphibian hibernation site should not be scheduled or undertaken between November and February 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 and ensure their safety.

37. The implementation of the mitigation strategy detailed in this report should be undertaken with due regard and consideration for the potential presence of other species at or neighbouring the development site.

38. **The mitigation strategy proposed does not allow GCN to be captured or removed from the site and released at another location.** In the unlikely event that GCN are found within the development site work must stop and the appointed ecologist and/or Natural England immediately contacted for advice on how to proceed.

7.3 Natural England GCN guidance relating to the licensed and non-licensed use of TAF states:

“Natural England cannot tell you whether to erect TAF, whether to apply for a licence, ‘approve’ the installation of TAF without a licence or whether any offences would be committed by doing so. It is for the person in charge of the scheme, normally through their ecological consultant, to decide on these matters’. Natural England indicates that ‘in only very limited circumstances, is it feasible that installing TAF would probably not result in an offence, and therefore no licence would be required. This could include cases where both the following criteria are met:

- *There is no habitat suitable for shelter or protection in the area subject to potentially harmful activities.*
- *The layout of the TAF would not result in substantial interference to the dispersal routes of great crested newts.*

The chance of an offence being committed increases close to breeding ponds and suitable habitats that can be used as hibernation or daytime refuge sites. For example, no offence is likely if the area to be fenced were a small tarmac car park, supporting no resting place habitat and not situated in between a breeding pond and key terrestrial habitat patches”.

7.4 In respect of the proposed development at the land to the north of The Elms, Darland Lane, Rossett terrestrial habitats to be directly impacted by the proposed development works (predominantly amenity grassland, hard-standing

and a building) were assessed by Amenity to offer GCN with shelter opportunities and foraging and dispersal habitat, categorised by Amenity, as predominantly 'Below Average'.

7.5 The development site is not situated between a breeding pond and key terrestrial habitat patches. In this instance, the short term installation of TAF at the development site would, in the view of Amenity, not result in substantial interference to the dispersal routes of great crested newts and therefore meets the very limited circumstances criteria as defined by Natural England.

7.6 The implementation of specific habitat enhancement measures, habitat features and monitoring specifically for GCN/amphibians is considered appropriate in relation to the proposed development. The proposed measures, features and monitoring are detailed below.

7.7 Proposed Habitat Enhancement Measures, Features & Monitoring:

1. Habitat connectivity measures will be incorporated into the development and will help to maintain links with the wider area. The connectivity measures proposed include; the retention/enhancement of any existing field margins and boundary hedgerows/vegetation with additional native species planting, as practicable and deemed appropriate by the appointed ecologist.
2. Any required gapping up of retained boundary hedgerow/s will be undertaken using a number of UK native species of local provenance such as; common hawthorn (*Crataegus monogyna*), field maple (*Acer campestre*), alder (*Alnus glutinosa*), hazel (*Corylus avellana*), crab apple (*Malus sylvestris*), field rose (*Rosa arvensis*) wild cherry (*Prunus avium*), holly (*Ilex aquifolium*) or guelder rose (*Viburnum opulus*). This will help to maintain and strengthen existing wildlife dispersal/flight corridors and increase foraging, shelter and nesting/roosting opportunities for a wide variety of species.
3. A single amphibian log pile refuge (minimum 1m x 2m x 1m high/deep, and left uncompacted) will be incorporated into the new development to provide hibernation habitat for any GCN using this site post-development. The construction of the log pile will follow NE guidelines, however it is acceptable that this feature be allowed to be buried within the ground as long as access

into the log pile refugia is available to GCN, e.g. by installing a drainage pile into the pile and leaving an 'open end' to the pipe.

4. Future habitat/enhancement management works at/neighbouring the site will be informed by current best practice relating to the long term management of GCN aquatic and terrestrial habitats as detailed in the EN GCN MG and/or The Great Crested Newt Conservation Handbook.
- 7.8 The developer/land owner will be responsible for adequately resourcing and ensuring the successful implementation of the GCN mitigation and monitoring strategy and habitat enhancement measures detailed above. Appropriate resources will be made available by the developer/landowner to ensure that the required future habitat management and GCN monitoring works at the site are successfully delivered.

8.0 CONCLUSIONS

- 8.1 Terrestrial habitats at the proposed development site to be directly impacted by the proposed development were assessed by Amenity as offering GCN with limited shelter opportunities and foraging or dispersal habitat categorised by Amenity as predominantly 'Below Average'. Consequently it has been concluded that GCN are reasonably unlikely to be present within the areas to be directly impacted by the development.
- 8.2 No GCN breeding sites, other water bodies or aquatic habitat will be adversely impacted as a consequence of the proposed development. The proposed development will result in some partial destruction, temporary disturbance and/or temporary destruction, followed by reinstatement of a relatively small area of intermediate and distant terrestrial habitat associated with Water body 1 and/or other ponds water bodies in the local area.
- 8.3 The predicted development effect and scale of impact has been assessed, with reference to the English Nature Great Crested Newt Mitigation Guidelines 2001 and, in the absence of appropriate mitigation measures, a 'low' negative development impact is predicted for the GCN population/s identified to be associated with Water body 1.

- 8.4 It has been concluded that the unmitigated commencement of development at the site could potentially have adverse impacts on individual GCN and/or other amphibians in the unlikely event that they are present or inadvertently stray into the working area following the commencement of development and take cover under/within temporary shelter habitat incidentally created as a consequence of the works.
- 8.5 Following careful consideration of the survey findings and given the predicted 'low' negative development impacts, the legal protection afforded to the species and current guidance issued by Natural England in respect of low impact development projects, Amenity have concluded that the RAM and habitat enhancement measures proposed and detailed in this report are in accordance with current Natural England guidance and that their successful implementation will effectively minimise the risk of breaching current legislation relating to GCN and will ensure that the favourable conservation status of the GCN population/s identified to be associated with Water body 1 is maintained within their natural range.
- 8.6 The status of GCN/amphibians at the site level has been reliably established, and the predicted development effect and scale of impact have been assessed and appropriate mitigation and habitat enhancement measures have been formulated. It should therefore be possible for 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, relating to GCN and biodiversity, by the implementation of planning condition/s when determining the planning application submitted in respect of the proposed development.

9.0 REFERENCES

Baker, J., Beebee, T., Buckley, J., Gent, T. & Orchard, D. (2011) Amphibian Habitat Management Handbook. Amphibian and Reptile Conservation, Bournemouth.

English Nature (2001) *Great Crested Newt Mitigation Guidelines*. Peterborough: English Nature.

Grundy, J. (2007) *Newts in your Pond and Garden*. Buxton Press Ltd.

Langton, T.E.S., Beckett, C.L., and Foster, J.P. (2001) Great Crested Newt Conservation Handbook. Froglife, Halesworth.

Oldham *et al.* (2000) *Evaluating the suitability of habitat for the Great Crested Newt (Triturus cristatus)*. Herpetological Journal 10(4), 143-155.

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 <i>Wildlife and Countryside Act</i> , 1981 (as amended)	No
Common toad	Provision 5 of Section 9 of the <i>Wildlife and Countryside Act</i> , 1981 (as amended) Section 41 of the <i>Natural Environment and Rural Communities (NERC) Act</i> , 2006	Yes
Great crested newt	Regulation 41 of <i>The Conservation of Habitats and Species (Amendment) Regulations</i> , 2012 Section 9 of the <i>Wildlife and Countryside Act</i> , 1981 (as amended) Section 41 of the <i>Natural Environment and Rural Communities (NERC) Act</i> , 2006	Yes
Smooth newt	Provision 5 of Section 9 of the <i>Wildlife and Countryside Act</i> , 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:
 - o to impair their ability to survive, to breed or reproduce, or to rear or nurture their young; or
 - o to impair their ability, in the case of animals of a hibernating or migratory species, to hibernate or migrate;
 - o 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:

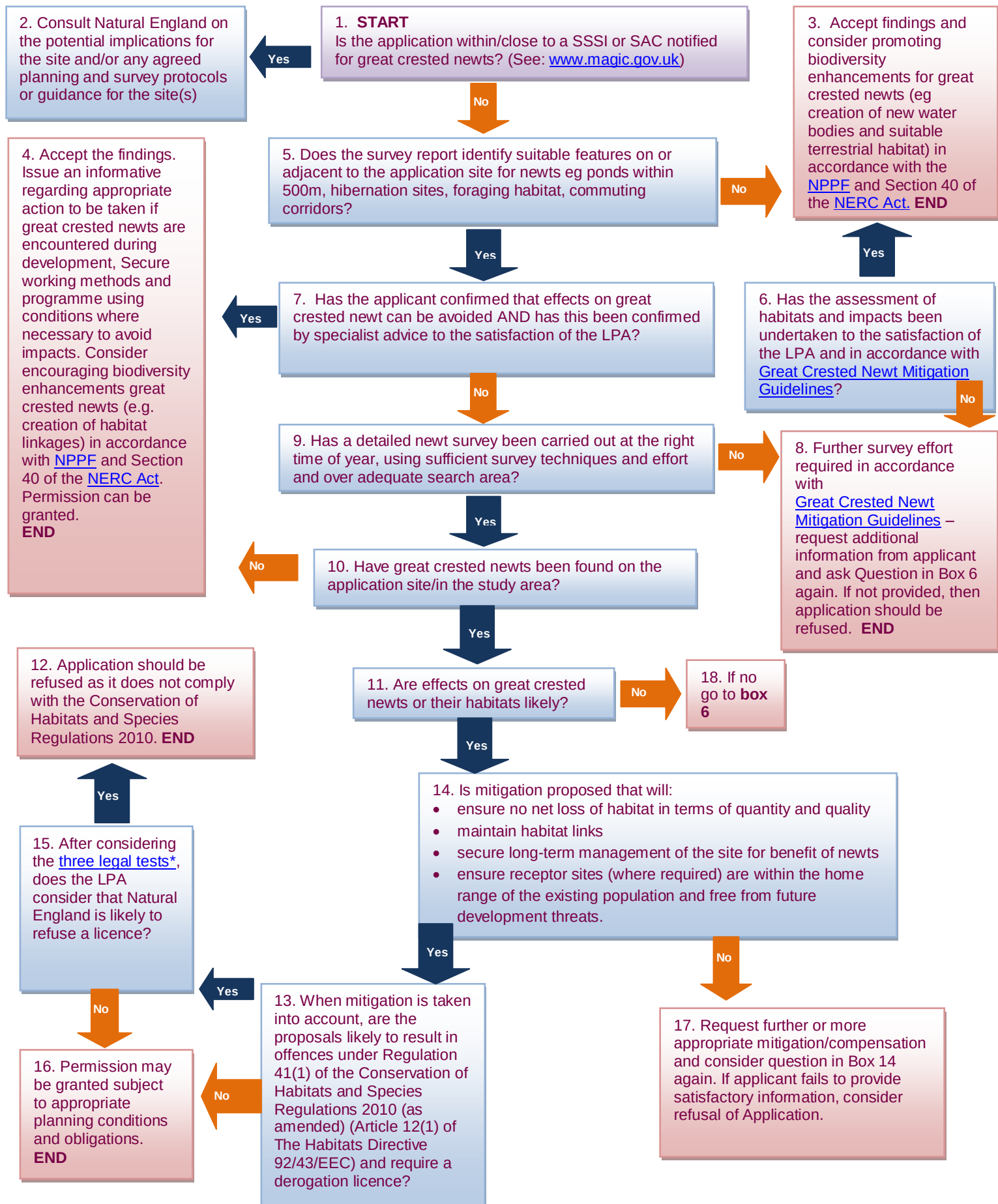
- Every public authority must, in exercising its functions, have regard, so far as is consistent with the proper exercise of those functions, to the purpose of conserving biodiversity;
- In complying with the above, a Minister of the Crown, government department or the National Assembly for Wales must 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.

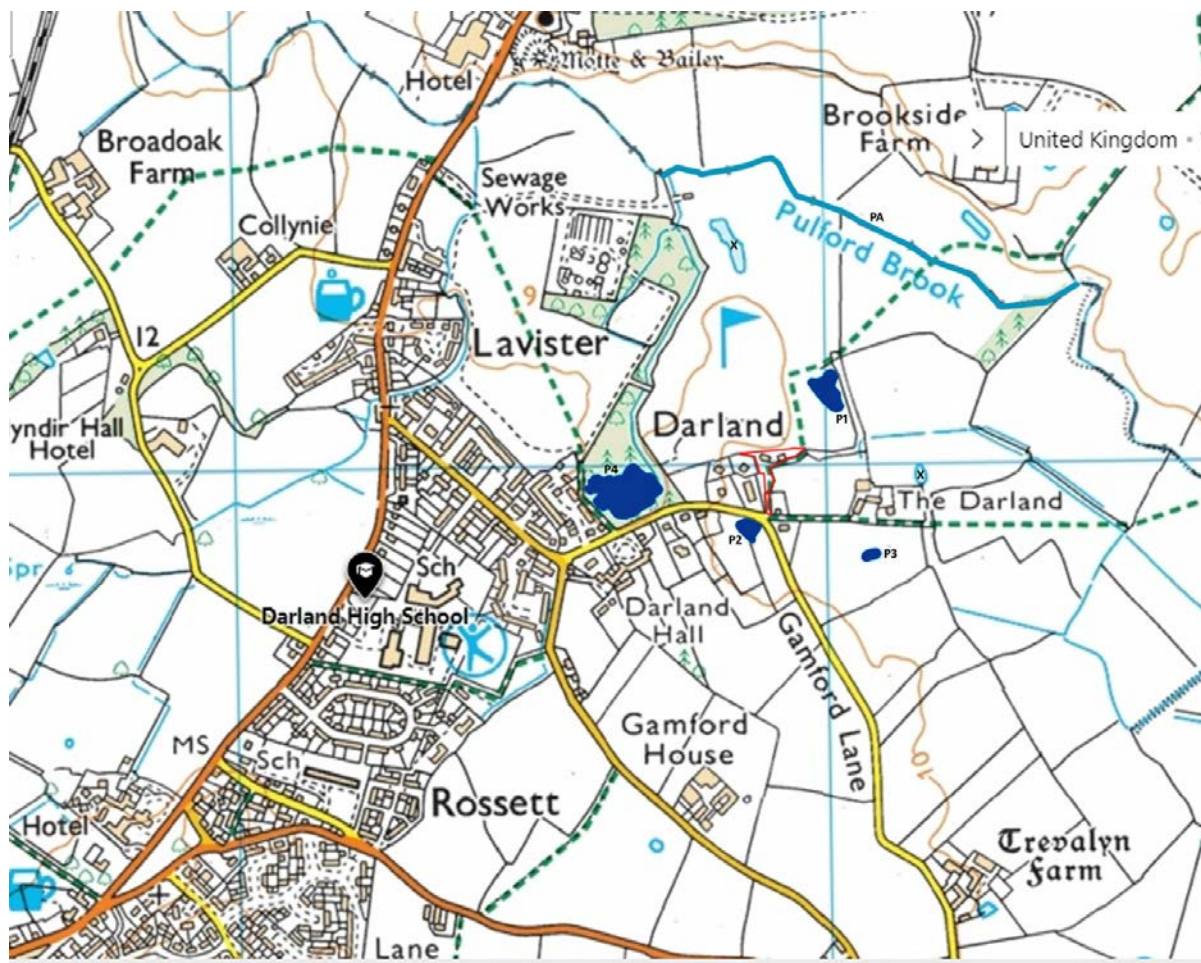
Appendix C: Natural England GCN Standing Advice Sheet Flow Chart

Appendix D: Site Location Plan (OS)

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)





Key:

- Site boundary
- Water bodies within 250m of site
- Water bodies 250m - 500m from site boundary
- X** Pond thought to be extinct (from recent aerial mapping)

Appendix E: Photographic Plates



Plate 1: View of the concrete apron that covers much of the proposed development site



Plate 2: Amenity grassland within the proposed development site



Plate 3: View of the spoil heap present on the proposed development site



Plate 4: Pond 1 is located approximately 110 metres to the north-east of the proposed development site



Plate 5: GCN were detected in Pond 1 during the 2018 GCN eDNA Survey



Plate 6: View of the proposed development site from Pond 1, showing expanse of arable land between the two areas

Appendix F: Land HIS (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

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.

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.

Version Control

Document date: 30 September 2014

Version Number: 1.1

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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 cristatus*) 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 handling 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

2.6.1 How 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 room¹.
- **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.

2.6.2 Appropriate 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 'suited-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.

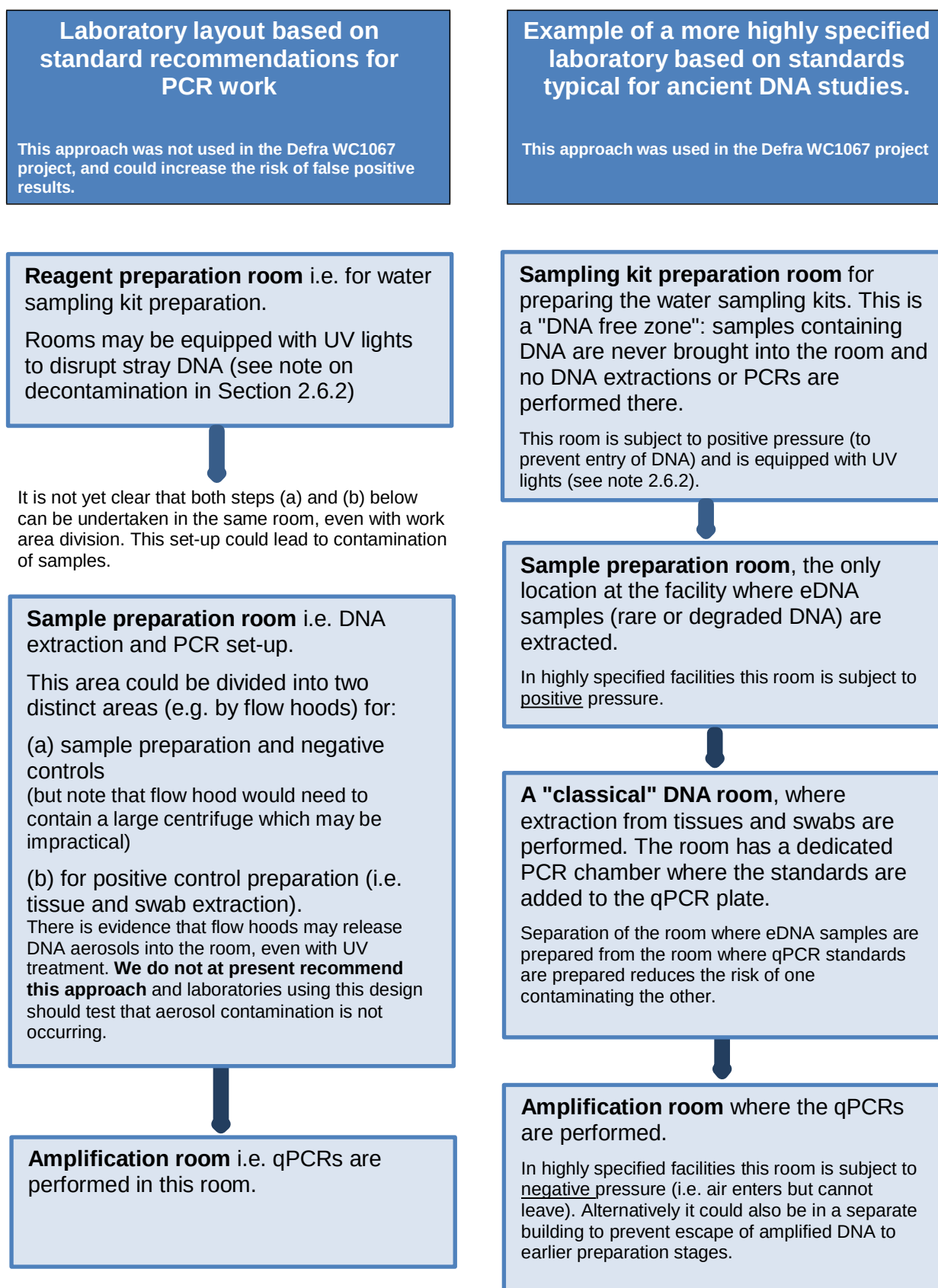
To reduce the risk of DNA contamination regular bleaching of the laboratory should be undertaken weekly. qPCR work should be undertaken inside a cabinet with UV light and in a room which is also lit by UV light outside the cabinet; to control aerosol DNA. Although UV lights are widely recommended for 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.

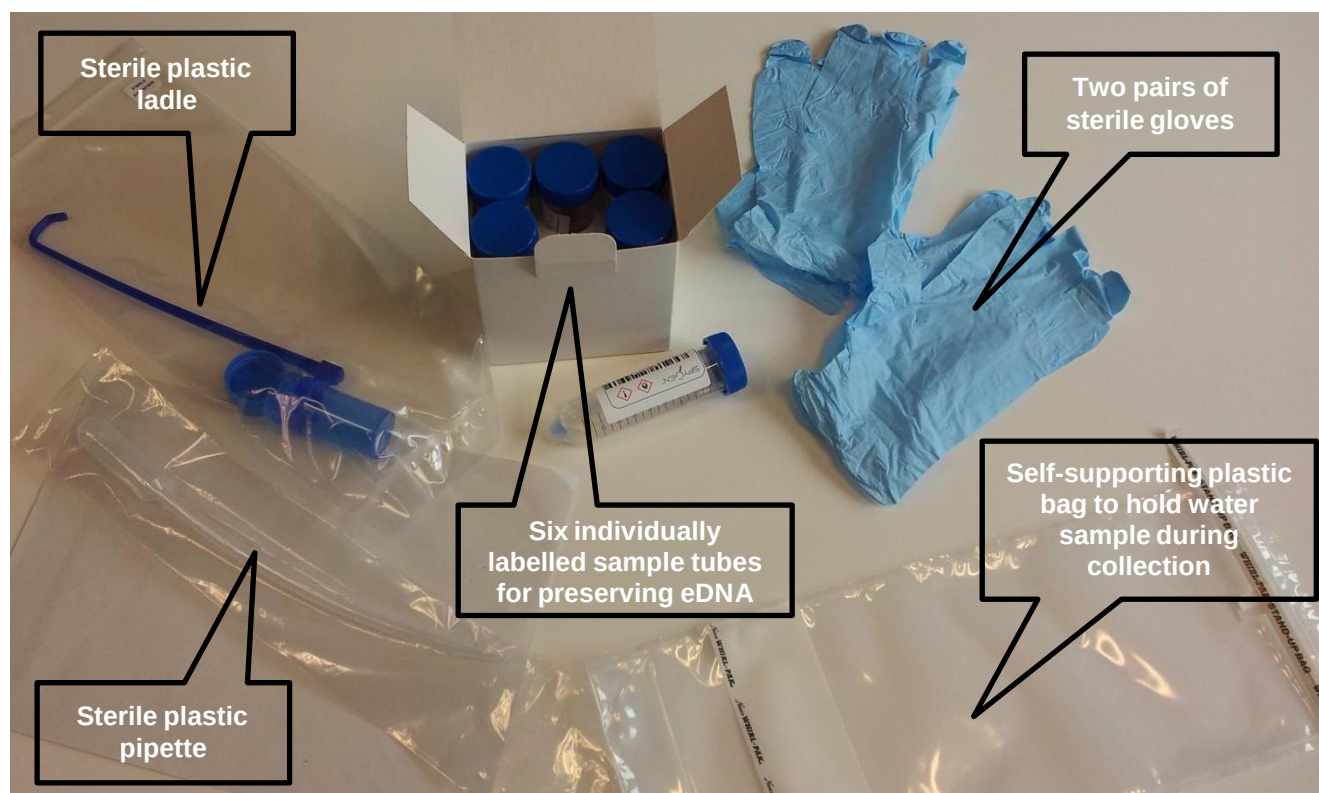
A single visit to the target pond should be made between mid-April and June, during the newt breeding season. eDNA samples can be collected at any time of day and in any reasonable weather conditions, including light rain. It may be best to avoid heavy rain as this makes sampling more difficult and might 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-Pak 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 mitochondrial 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 $14000 \times g^1$, 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 subsamples, 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

¹The centrifugation speed suggested originally by Ficetola et al. (2008) was $5500 \times g$. Internal tests made by Spygen indicated that better results were found with the highest centrifugation speed, which led to the adoption of $14,000 \times g$ 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 crested 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 to the sample and running qPCR in duplicate. If a different than expected C_t^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 ddH₂O
- 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 McKinneil (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.

Specific thanks for technical advice on the preparation of this document are given to Neil Boonham (FERA), Simon Creer (Bangor University), Helen Rees (ADAS) and Kerry Walsh (Environment Agency), and to Mike Wilkinson and Katharine Woods (both of Natural England).

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

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

Folio No: E3268
Report No: 1
Order No: 0027
Client: DAWN GOODFELLOW
Contact: Dawn Goodfellow
Contact Details: dawn_goodfellow@hotmail.com
Date: 20/06/2018

TECHNICAL REPORT

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

Date sample received at Laboratory: 12/06/2018
Date Reported: 20/06/2018
Matters Affecting Results: None

RESULTS

Lab Sample No.	Site Name	O/S Reference	SIC	DC	IC	Result	Positive Replicates
1801	Darland Lane	SJ 38010 58130	Pass	Pass	Pass	Positive	12

SUMMARY

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

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

RESULTS INTERPRETATION

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

Site Name- Information on the pond.

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

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

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

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

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

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

METHODOLOGY

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

The analysis is conducted in two phases. The sample first goes through an extraction process where all six tubes are pooled together to acquire as much eDNA as possible. The pooled sample is then tested via real time PCR (also called q-PCR). This process amplifies select part of DNA allowing it to be detected and measured in 'real time' as the analytical process develops. qPCR combines PCR amplification and detection into a single step. This eliminates the need to detect products using gel electrophoresis. With qPCR, fluorescent dyes specific to the target sequence are used to label PCR products during thermal cycling. The accumulation of fluorescent signals during the exponential phase of the reaction is measured for fast and objective data analysis. The point at which amplification begins (the Ct value) is an indicator of the quality of the sample. True positive controls, negatives and blanks as well as spiked synthetic DNA are included in every analysis and these have to be correct before any result is declared so they act as additional quality control measures.

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

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

different buildings at our premises for added

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

Reported by: Sam Humphrey

Approved by: Derry Hickman

End Of Report
