



## Preliminary Ecological Appraisal

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Houghton Main Colliery  
for:

**Peel Environmental Ltd**

CRM.066.006.EC.R.001



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## Non-Technical Summary

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- i. In June 2016 Enzygo Ltd was commissioned by Peel Environmental Ltd. to undertake a Preliminary Ecological Appraisal (PEA) at land off Houghton Main Colliery Roundabout, Park Spring Road S72 7LG (central grid reference: SE 41693 06444), located within the Barnsley Council planning authority. This study will form the full planning permission for the proposals for the development of a Waste Resource Recovery Centre, which will treat up to 150,000 tonnes per annum of waste through a gasification process which will export over 20MW renewable electrical power.
- ii. The following ecological constraints and associated recommendations to avoid/ mitigate/ compensate for potential impacts have been identified:
  - **Deciduous Woodland and Hedge Habitat of Principal Importance (HPI)** (immediate vicinity of site)- avoid indirect impacts to root zones during construction;
  - **Bats** (suitable features off-site and low habitat suitability on-site) avoid lighting impacts to off-site habitats, and undertake bat activity survey of site;
  - **Birds** (general nesting potential within hedgerows and scrub) – Avoid disturbance of nesting birds through sensitive site clearance outside nesting period OR Ecological Clerk of Works (ECoW) checks;
  - **Common Reptiles** (potential for common species)- Undertake a reptile survey;
  - **England Species of Principal Importance (SPI)/Local Biodiversity Action Plan (BAP) and Notable Mammal and Invertebrate Species** (Hedgehog and invertebrate species recorded in area) – Allow to disperse into surrounding habitats during site clearance.

# 1 Introduction

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## 1.1 Commission

- 1.1.1 In June 2016 Enzygo Ltd was commissioned by Peel Environmental Ltd. to undertake a Preliminary Ecological Appraisal (PEA) at land off Houghton Main Colliery Roundabout, Park Spring Road S72 7LG (central grid reference: SE 41693 06444), located within the Barnsley Council planning authority. This study will form the full planning permission for the proposals for the development of a Waste Resource Recovery Centre, which will treat up to 150,000 tonnes per annum of waste through a gasification process which will export over 20MW renewable electrical power.
- 1.1.2 The proposed works will involve the construction of a Waste Resource Recovery Centre with associated buildings including sub-station, weighbridge and parking facilities. All boundary features i.e. hedgerows, woodland and disused railway corridor will be retained. All waste will be delivered and stored indoors, there will be no discharge into the local water course, and a sensitive lighting scheme will be incorporated to avoid lighting impacts to surrounding habitats. Additionally, environmental assessments (Enzygo, 2016) have identified that there will be no significant increase in: dust; air pollution; or noise during operational activities. Refer to Appendix A for proposed development plan.

## 1.2 Aims and Objectives

- 1.2.1 The aim of the survey and supporting desk study was to satisfy the requirements of the National Planning Policy Framework (NPPF), identifying ecological features within or near the site that could potentially pose a constraint to the proposed development and opportunities for incorporating biodiversity enhancements into the development proposals. The following ecological features are relevant to this exercise:
- Statutory sites designated or classified under international conventions or European legislation;
  - Statutory sites designated under national legislation (excluding geological);
  - Locally designated wildlife sites;
  - Ancient Woodland Inventory sites, Important Hedgerows (as defined by The Hedgerow Regulations 1997), Veteran Trees, trees listed under Tree Preservation Orders (TPOs), and trees within a Conservation Area;
  - England Habitats of Principal Importance (HPI) identified as requiring action in the UK Biodiversity Action Plan (UK BAP) and Local BAP Habitats;
  - Legally protected species;
  - England Species of Principal Importance (SPI) identified as requiring action in the UK BAP and Local BAP Species;
  - Notable species (which includes: Species of conservation concern and Red Data Book (RDB) species, Birds of Conservation Concern (BOCC), and nationally rare and nationally scarce species);
  - Invasive species (listed under section 14 of Schedule 9); and
  - The wider green infrastructure resource.

1.2.2 This report has been produced with reference to current guidelines for preliminary ecological appraisal (CIEEM, 2012) and in accordance with BS42020:2013: Biodiversity – Code of Practice for Planning and Development (BSI, 2013).

1.2.3 This report remains valid for 2 years from the date of publication, or until conditions across the site should substantially alter.

### 1.3 Background

1.3.1 The site was previously used for open cast mining up to 2001. The land was reclaimed and compacted to provide a platform suitable for industrial development. Since the cessation of works a valid re-use of the site has been sought. The planning permission history covering the proposed development site includes:

- **2015/0137**; Erection of a Renewable Energy Park comprising of a Timber Resource Recovery Centre and associated infrastructure (Approved Subject to Legal Agreement);
- **2011/1443**; Erection of 19 industrial units with associated external works and landscaping (Extension to time limit of application 2008/1426) (Approved with Conditions); and
- **2008/1426**; Erection of 19 industrial units with associated external works and landscaping (Approved).

1.3.2 To date, there have been no discussions with the County Ecologist in regards to this application and no historical ecological reports have been consulted.

### 1.4 Site Context

1.4.1 The approximately 3.5Ha site is located south of Grimethorpe, South Yorkshire, and at the far south-eastern point of the former Houghton Colliery. It is comprised of unmanaged poor semi-improved grassland with colonising scattered scrub. Planted hedgerows and hedgerows with trees delineate the site boundaries. The site is further bordered by a dismantled colliery railway to the north and the A4195 (Park Spring Road) to the east.

Figure 1.1: Surveyed Area



*Image courtesy of Google Earth Pro 7.1.5.1557, 53°33'09.73"N 1°22'21.13"W Elev.34m. Imagery date 21<sup>st</sup> September 2008. Image sourced 12<sup>th</sup> August 2016.*

1.4.2 The site lies within the Nottinghamshire, Derbyshire and Yorkshire Coalfield, “A generally low-lying area, with hills and escarpments above wide valleys, the landscape embraces major industrial towns and cities as well as villages and countryside” (Natural England, 2016). Over half of the national Character Area (NCA) is designated as greenbelt land.

## 2 Methodology

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### 2.1 Desk Study

2.1.1 Desk study details were obtained from the following sources on the associated dates to provide background on ecological features in the vicinity of the site. Records over 10 years old for transient species and all species protected from sale only are excluded. In each case the search included the site and the specified area beyond the site boundary. The search radius was based on the professional judgement of the ecologist leading the appraisal, taking into account the scope of the proposed works and associated potential impacts, with reference to current guidelines for preliminary ecological appraisal (CIEEM, 2013). Records obtained included:

- European statutory sites within a 15km radius, national statutory sites designated for bats and birds within a 10km radius, all other national statutory sites within a 2km radius, and England HPI identified as requiring action in the UK BAP (JNCC, 2015) and Ancient Woodland within a 0.5km radius (Natural England GIS Digital Boundary Database and Natural England Site Designations, on 10<sup>th</sup> August 2016);
- TPOs and Conservation Areas within the immediate zone of influence (Barnsley Council 10<sup>th</sup> August 2016);
- Waterbodies within a 0.5km radius (Online mapping sources including: Google Maps; MAGIC; and Ordnance Survey Street View, 11<sup>th</sup> August 2016); and
- Locally designated wildlife sites, Legally protected species, England SPI identified as requiring action in the UK BAP (JNCC, 2015), Barnsley Local BAP Habitats/Species, any Notable species (which includes: Species of conservation concern and RDB species (JNCC, 2014a), BOCC (Eaton et al., 2015); and nationally rare and nationally scarce species (JNCC, 2014b)) and Invasive species within a 0.5km radius, and important hedgerows/veteran trees within the immediate zone of influence (Barnsley Biological Records Centre (BBRC), 10<sup>th</sup> August 2016).

### 2.2 Field Survey

#### *Phase I Habitat Survey*

- 2.2.1 The Extended Phase I Habitat Survey was undertaken on 27<sup>th</sup> June 2016 by a Consultant Ecologist from Enzygo (Kirsty Rogers MZool (Hons), Grad CIEEM) who satisfies all necessary field survey competencies as stipulated by the Chartered Institute for Ecology and Environmental Management (CIEEM). Weather conditions on the day of survey were dry, with 90% cloud cover and a light wind. The temperature was 14°C.
- 2.2.2 Phase I Habitat Survey (JNCC, 2010) is a standard technique for obtaining baseline ecological information for large areas of land in which the main vegetation types present within the survey area are mapped using a standard set of habitat categories.
- 2.2.3 In addition to mapping, each of the main habitats within the survey area was described; including details of component plant species abundances (recorded using the DAFOR scale: D=Dominant, A=Abundant, F=Frequent, O=Occasional, R=Rare). Target Notes, detailing specific features of interest, have also been included.
- 2.2.4 Incidental observations of Legally protected species, England SPI/Local BAP Species, any Notable species (which includes: Species of conservation concern and RDB species; BOCC; and nationally rare and nationally scarce species) and Invasive species, and the potential for such species to

occur on site (and in the surrounding landscape where relevant) were also noted; however, no specific species surveys were undertaken.

#### *GCN eDNA survey*

- 2.2.5 Given the sites location in proximity to several waterbodies which could support populations of Great Crested Newt (GCN) (*Triturus cristatus*), initial consultation with the client recommended an assessment of all waterbodies within a 250m radius. In this instance, in accordance with current guidance (English Nature, 2001) the search radius was not extended out to 500m due to sufficient waterbodies present within 250m and supporting terrestrial habitat. A GCN eDNA assessment was identified as the most appropriate survey method, which is an accepted methodology to determine the presence/absence of GCN (Natural England, 2015). Water samples were taken on the 28<sup>th</sup> June 2016 by a Consultant Ecologist from Enzygo (Kirsty Rogers MZool (Hons), Grad CIEEM) who satisfies all necessary field survey competencies as stipulated by the Chartered Institute for Ecology and Environmental Management (CIEEM), and who is accredited under Natural England CL08 (Class 1) survey licence 2015-19227-CLS-CLS. Refer to Appendix B for detailed methodology.

## **2.3 Assessment**

- 2.3.1 Potential ecological constraints to development have been identified from desk study and field survey data and in the absence of a proposed site layout, assumes complete site clearance. An ecological value has been assigned to each ecological feature that poses a constraint/potential constraint to development in accordance with the Chartered Institute of Ecology and Environmental Management (CIEEM) guidelines (CIEEM, 2016), where sufficient baseline data are available to do so. Where no value has been assigned, this is due to insufficient information.
- 2.3.2 An assessment of likely ecological impacts has been undertaken in accordance with CIEEM guidelines (CIEEM, 2016) only where clear evidence is available to substantiate and justify the findings. In the absence of such evidence, the ecological feature is merely identified as a potential constraint to development.
- 2.3.3 Where ecological constraints to development are identified, further survey requirements and/or avoidance, mitigation, compensation measures that are proportionate to the predicted degree of risk to biodiversity and to the nature and scale of the proposed works are described. In addition, in accordance with the NPPF, opportunities to enhance or create benefits to wildlife are explored alongside the hierarchy of aforementioned measures.

## **2.4 Nomenclature**

- 2.4.1 The English names of flora and fauna species are given in the main text of this report. Scientific names are used alongside the English name where this first occurs. Vascular plants and Charophytes follow the nomenclature of The Botanical Society for the British Isles database (BSBI, 2007) with all other flora and fauna following the National Biodiversity Network (NBN) Gateway (NBN, 2014).

## **2.5 Limitations**

- 2.5.1 This document does not contain a comprehensive list of botanical species on site. Only plant species characteristic of each habitat and incidental observations of notable plant species were recorded. In addition, many plant species are only evident at certain times of the year and so some plant species may have gone undetected.

- 2.5.2 Data held by consultees may not be exhaustive. The absence of evidence, does not indicate evidence of absence.
- 2.5.3 Enzygo cannot take responsibility for the accuracy of external data sources and as such discrepancies and inaccuracies may occur.
- 2.5.4 Natural England do not hold information on Ancient Woodland less than 2Ha in size.
- 2.5.5 BBRC do not hold information on important hedgerows/veteran trees.

### 3 Results and Evaluation

#### 3.1 Sites and Habitats Identified by the Desk Study

3.1.1 Sites and habitats identified by the desk study are presented below, along with the reason(s) for their designation and associated ecological value. Potential direct or indirect constraints resulting from the construction phase or operation of the site have been identified, including details of any Impact Risk Zone (IRZ) within which the proposed works fall. Where potential constraints have been identified, the site/habitat is shown on Drawing CRM.066.006.EC.D.001.

**Table 3-1: Sites and Habitats Identified by the Desk Study**


Name (distance/direction from works)	Details and Ecological Value	Potential Constraint
<b>Statutory sites designated or classified under international conventions or European legislation</b>		
None	-	-
<b>Statutory sites designated under national legislation</b>		
Denaby Ings Site of Special Scientific Interest (SSSI) 9.4km SE	Denaby Ings represents one of the most diverse wetlands in the county and is notable for its breeding bird community of riparian and wader species. This site is of National value.	No- No direct or indirect impacts perceived (no similar habitats on site).
West Haigh Wood Local Nature Reserve (LNR) 1.6km N	This site is comprised of ancient woodland and young broad-leaved woodland and supports a number of UK BAP bird species. This site is of National value.	No- No direct or indirect impacts perceived (no similar habitats on site).
Proposed works are located within IRZ of SSSI beyond 2km.	Threshold criteria only require consultation with Natural England for "Airports, helipads and other aviation proposals".	No
<b>Locally designated wildlife sites</b>		
Edderthorpe Ings Local Wildlife Site (LWS) 108m W&N	The site is comprised of open water, formed on formerly drained agricultural land, areas of swamp, marshy grassland and neutral grassland habitats are also present. This site also includes the banks of the River Dearne and is important for a range of bird species but particularly Schedule 1 species: Avocet ( <i>Recurvirostra avosetta</i> ) and Little-ringed Plover ( <i>Charadrius dubius</i> ). This site is of County value.	No – No direct or indirect impacts (LWS upstream of works, no significant increase in dust, air pollution or noise, and sensitive lighting scheme to be incorporated).



Name (distance/direction from works)	Details and Ecological Value	Potential Constraint
<b>England HPI, Local BAP Habitats, Ancient Woodland, Important Hedgerows, Veteran Trees, TPOs and Conservation Areas</b>		
Deciduous Woodland HPI, <5m from site boundary, N.	HPI are of National value.	Yes- a) potential works within root zones during construction activities/site clearance. Significant adverse, permanent, reversible impact.


### 3.2 Habitats Identified on Site

3.2.1 The following habitats were identified on site during the course of the field survey. The distribution of these habitats is shown on Drawing CRM.066.006.EC.D.001.

**Table 3-2: Habitat Identified on Site**

Habitat Type (code)	Details and Ecological Value	Potential Constraint
Poor Semi-Improved Grassland (B6) 	<p>The majority of the site is comprised of poor semi-improved grassland. This habitat is unmanaged with grasses reaching 1m in places, and is becoming lost to successional scrub (see below). Grass species present include dominant Cock’s-foot Grass (<i>Dactylus glomerata</i>), Annual Meadow-grass (<i>Poa annua</i>), Perennial Rye Grass (<i>Lolium perenne</i>), Creeping Bent (<i>Agrostis stolonifera</i>) and False Oat-grass (<i>Arrhenatherum elatius</i>) with occasional Timothy Grass (<i>Phleum pratense</i>) and Reed Canary Grass (<i>Phalaris arundinacea</i>) also present.</p> <p>Herb species found within include abundant Common Dandelion (<i>Taraxacum officinale</i>), Creeping Thistle (<i>Cirsium arvense</i>), White Clover (<i>Trifolium repens</i>), Rosebay Willowherb (<i>Chamerion angustifolium</i>) and Broad-leaved Dock (<i>Rumex obtusifolius</i>). Occasionally abundant species include Birds-foot Trefoil (<i>Lotus corniculatus</i>), Common Vetch (<i>Vicia sativa</i>), Ribwort Plantain (<i>Plantago lanceolate</i>), Common Ragwort (<i>Jacobaea vulgaris</i>), Lesser Trefoil (<i>Trifolium dubium</i>) and Oxeye Daisy (<i>Leucanthemum vulgare</i>). Common Spotted Orchid (<i>Dactylorhiza fuchsia</i>) is found rarely to the south of the site.</p> <p>Towards the centre of the site the grassland is wetter with small ephemeral pools forming, Pedunculate Sedge (<i>Carex pedunculata</i>), Common Reedmace (<i>Typha latifolia</i>) and moss species are found frequently, with rarely occurring Northern Marsh Orchid (<i>Dactylorhiza purpurella</i>) also found here.</p>	No – Loss of this habitat alone is not perceived to be significant.

Habitat Type (code)	Details and Ecological Value	Potential Constraint
	<p>(This habitat does not meet the criteria definition of Acid/Neutral Flush, which is categorised as minerotrophic mires and habitat associated with water movement, which typically support species-poor vegetation including Sphagnum carpet overlain with Carex and/or Juncus species.</p> <p>Not a HPI or listed as a Local BAP habitat. Within zone of influence value only.</p>	
<p>Scattered Scrub (A2.2)</p> 	<p>Encroaching scattered scrub comprised of dominant Silver Birch (<i>Betula pendula</i>), with occasionally occurring Ash (<i>Fraxinus excelsior</i>), Goat Willow (<i>Salix caprea</i>), Pedunculate Oak (<i>Quercus robur</i>) and rarely occurring Hawthorn (<i>Crataegus monogyna</i>) scattered throughout.</p> <p>Not an HPI or Local BAP Habitat. Within zone of influence value only.</p>	<p>No – Loss of this habitat alone is not perceived to be significant.</p>
<p>Hedgerow Species-Poor (J2.1.2)</p> 	<p>H1 is a planted hedgerow extending 4-5m in width and approximately 4m in height. The understory is patchy with species of poor semi-improved grassland present and areas of exposed weed control membrane. Silver Birch and Hawthorn is abundantly present, with frequently occurring Dog Rose (<i>Rosa canina</i>) and Goat Willow. Occasionally present Pedunculate Oak and rarely occurring Bramble (<i>Rubus fruticosus</i>) also present.</p> <p>H2 is a planted hedgerow comprised of Hawthorn and Blackthorn (<i>Prunus spinose</i>). This habitat is not managed, and stands at 1.2m in height.</p> <p>Hedgerows, for the purpose of HPI and Local BAP classification, are defined as any boundary line of trees or shrubs over 20 metres long and less than 5 metres wide where any gaps between the trees or shrubs are less than 20 metres wide. These must also be comprised of 80% or more of at least 1 woody UK native species. H1 and H2 meet the criteria to warrant classification as HPI and Local BAP Habitat. May also meet criteria to be classified as 'Important' under the hedgerow regulations. Boundary hedgerows are to be retained with existing breaches utilised.</p> <p>This habitat is of County value.</p>	<p>Yes- a) degradation from works within root zones during construction activities.</p> <p>Minor adverse, permanent, reversible impact.</p>

Habitat Type (code)	Details and Ecological Value	Potential Constraint
<p>Hedgerow (with trees) Species-poor (J2.3.2)</p> 	<p>H3 is an unmanaged “leggy” hedgerow standing at 5-6m in height with a post and barbed-wire fence within. This hedgerow is comprised of dominant Silver Birch with frequent Ash, Goat Willow, Pedunculate Oak and Hazel (<i>Corylus avellana</i>). Occasionally occurring Hawthorn, Blackthorn and Dog Rose is also present. The understory is comprised of species similar to that found within poor semi-improved grassland elsewhere on site. Hedgerows, for the purpose of HPI and Local BAP classification, are defined as any boundary line of trees or shrubs over 20 metres long and less than 5 metres wide where any gaps between the trees or shrubs are less than 20 metres wide. These must also be comprised of 80% or more of at least 1 woody UK native species. H3 meets the criteria to warrant classification as HPI and Local BAP Habitat. May also meet criteria to be classified as ‘Important’ under the hedgerow regulations. Boundary hedgerows are to be retained with existing breaches utilised. This habitat is of County value.</p>	<p>Yes- a) degradation from works within root zones during construction activities. Minor adverse, permanent, reversible impact.</p>

### 3.3 Protected and Notable Species

3.3.1 The possibility that Legally protected species, England SPI/Local BAP Species, any Notable species (which includes: Species of conservation concern and RDB species; BOCC; and nationally rare and nationally scarce species), and Invasive species will pose a constraint to the proposed development is evaluated for each of the aforementioned based on assessment of habitat suitability and other relevant factors, such as: national distribution of each species/group; previous records of species occurrence obtained through the desk study; connectivity to suitable habitats in the surrounding landscape; field signs suggesting presence of species within or near to the site; and probability of the proposed works having an adverse impact on the species/group if present. Given the large number of England SPI/Local BAP Species, and Notable species (which includes: Species of conservation concern and RDB species; BOCC; and nationally rare and nationally scarce species), these have only been included if identified from the desk study and/or observed on site during the field survey.

**Table 3-3: Species Identified from the Desk Study and Field Survey**

Species/Group	Desk Study Record (distance/direction from works)	Field Survey Habitat/Evidence	Ecological Value	Potential Constraint
Bats ( <i>Chiroptera spp.</i> )	Two records of unidentified bats ( <i>Chiroptera spp.</i> ) within search radius, 2km E.	No buildings or structures on site. Trees present are too young/thin to provide opportunities for roosting bats. Grassland/hedgerows offer low suitability for commuting/foraging bats (Collins 2016), also partially lit from adjacent industrial buildings. Riparian corridor, dismantled railway, woodland and mature trees off-site offer higher quality bat habitat.	Further surveys required to determine value. All bat species listed as England SPI, and on Local BAP.	Yes – a) loss of low suitability habitat during site clearance/construction activities; b) potential indirect disturbance of any retained roosts and off-site commuting/foraging habitat, from lighting during construction or operation of site. Significant adverse, permanent/ temporary, irreversible/ reversible impact.
Badger ( <i>Meles meles</i> )	No records	No evidence of Badger setts on or within 30m of site boundary. General mammal runs present on western boundary.	-	No
Dormouse ( <i>Muscardinus avellanarius</i> )	No records	None (scattered scrub on-site fragmented, boundary hedgerows and off-site woodland offer more suitable habitat)	-	No
Otter ( <i>Lutra lutra</i> )	No records	None (River Dearne off-site to west offers suitable habitat)	-	No
Water Vole ( <i>Arvicola amphibious</i> )	7 records of Water Vole associated with the River Dearne, 60m W.	None (River Dearne off-site to west offers suitable habitat)	-	No
Other Protected Mammals	No records	None	-	No
Specially Protected Birds	Records of 56 species, including Whimbrel ( <i>Numenius phaeopus</i> ), Merlin ( <i>Falco columbarius</i> ), Kingfisher ( <i>Alcedo atthis</i> ) and Barn Owl ( <i>Tyto alba</i> ) the majority associated with Edderthorpe lngs, 108m W.	None (grassland too dense/scrubby for ground nesting, and unlikely to be used as secondary feeding ground by Schedule 1 birds recorded in area)	-	No

Species/Group	Desk Study Record (distance/direction from works)	Field Survey Habitat/Evidence	Ecological Value	Potential Constraint
All Other Birds	Record of 74 bird species, including Kittiwake ( <i>Rissa tridactyla</i> ), Lapwing ( <i>Vanellus vanellus</i> ), Skylark ( <i>Alauda arvensis</i> ) and Wigeon ( <i>Anas penelope</i> ) the majority associated with Edderthorpe Ings, 108m W.	Scrub and hedgerow offer suitable habitat for general nesting birds (grassland too dense/scrubby for ground nesting)	Species dependant. Within the zone of influence value only.	Yes – a) disturbance of nesting birds during site clearance. Minor adverse, temporary, reversible impact (insignificant impact from the loss of limited extent of habitat.
Common Reptiles	No records	Grassland, scrub and hedgerows provide cover and basking opportunities. Brash also provides hibernacula.	All Common Reptiles listed as England SPI but not as a Local BAP species. Within zone of influence value only.	Yes – a) risk of killing/injury during site clearance works; b) loss of approx. 3.5Ha of suitable habitat during site clearance works. Significant adverse, temporary/permanent, irreversible impact.
Great Crested Newt ( <i>Triturus cristatus</i> )	No records	No suitable breeding habitat on site (ephemeral pool dry most of year with no aquatic vegetation so does not provide suitable breeding conditions). Three waterbodies within a 250m radius (further 2 within a 500m radius but beyond River/Roads which are considered to act as barriers to the movement of amphibians). Results of eDNA test for all 3 ponds 'Negative' indicating absence of GCN. Refer to Appendix C for full details. Grassland, scrub and hedgerows provide suitable cover and foraging habitat. Brash also provides hibernacula.	-	No
Other Protected Herpetofauna	No records	None	-	No
White-clawed Crayfish	No records	None (River Dearne off-site to west offers suitable habitat)	-	No

Species/Group	Desk Study Record (distance/direction from works)	Field Survey Habitat/Evidence	Ecological Value	Potential Constraint
<i>(Austropotamobius pallipes)</i>				
Fish/Marine	No records	None (River Dearne off-site to west offers suitable habitat)	-	No
Protected Invertebrates	No records	Only common habitats present, unlikely to support protected invertebrates	-	No
Protected Flora	No records	Only common habitats present, unlikely to support protected plants	-	No
England SPI/Local BAP & Notable Mammal species	Records of Hedgehog ( <i>Erinaceus europaeus</i> ) 960m S	Grassland, scrub and hedgerows	Species dependant. Within zone of influence value only	Yes – a) risk of killing/injury during site clearance. Minor adverse, permanent, reversible impact.
England SPI/Local BAP & Notable Invertebrate species	Records of Small Heath ( <i>Coenonympha pamphilus</i> ) and Wall butterfly ( <i>Lasiommata megera</i> ) 775m N	Grassland, scrub and hedgerows	Species dependant. Within zone of influence value only	Yes – a) risk of killing/injury during site clearance. Minor adverse, permanent, reversible impact.
Invasive Flora	Records of Himalayan Balsam ( <i>Impatiens glandulifera</i> ) and New Zealand Pigmyweed ( <i>Crassula helmsii</i> ) within Edderthorpe Ings, 108m W	None (Himalayan Balsam noted immediately off-site to west along River Dearne corridor and New Zealand Pigmyweed found within pond off-site to the south)	-	No
Invasive Fauna	No records	None	-	No

### **3.4 Contribution to the Wider Green Infrastructure Resource**

- 3.4.1 The site forms part of a wider area of grassland and scrub that has established over the former Colliery, but is not a significant part of the wider green infrastructure resource, hence no impacts are perceived should this be lost. Habitats off-site to the north and west form a more significant part of the wider green infrastructure resource which is associated with the River Dearne corridor, disused railway and Edderthorpe Ings.
- 3.4.2 There is no aquatic connectivity on site. The closest tributary is the River Dearne off-site to the west.
- 3.4.3 The application site is not located within any known dark zones. However, it is likely to be classified within environmental zone E2 (Village or relatively dark outer suburban locations) which is defined as 'Low District Brightness'.

## 4 Relevant Legislation and Policy

### 4.1 Legislation

4.1.1 Wildlife legislation and policy relevant (or potentially relevant pending further survey) to the proposed works, based on the findings of the desk study and field survey are set out below. This legal information is a summary only, and the original legal documents should be consulted for definitive information.

**Table 4-1: Legislation Protection Afforded to Sites/Habitats that could Potentially be affected by the Proposed Works.**

Designated Site/Habitat	Legal Status
Hedgerows	Hedgerows that meet certain criteria are protected by The Hedgerows Regulations 1997, under which it is an offence to remove or destroy such hedgerows without permission from the Local Planning Authority.

**Table 4-2: Legislation Protection Afforded to Species that could Potentially be Affected by the Proposed Works.**

Species	Legal Status
European Protected	
Bats	<p>These animal species and their breeding sites or resting places are protected under Regulation 41 of the Conservation of Habitats and Species (Amendment) Regulations 2012, which makes it illegal to:</p> <ul style="list-style-type: none"> <li>• Deliberately capture, injure or kill any such animal or to deliberately take or destroy their eggs;</li> <li>• Deliberately disturb such an animal;</li> <li>• Damage or destroy a breeding site or resting place of such an animal.</li> </ul> <p>European Protected Species (EPS) licences can be granted by Natural England in respect of development to permit activities that would otherwise be unlawful under the Conservation Regulations, providing that the following 3 tests (set out in the EC Habitats Directive) are passed:</p> <ul style="list-style-type: none"> <li>• The development is for reasons of overriding public interest;</li> <li>• There is no satisfactory alternative; and</li> <li>• The favourable conservation status of the species concerned will be maintained and/or enhanced.</li> </ul> <p>Under Regulation 9(5) of the Conservation Regulations, Planning Authorities have a legal duty to 'have regard to the requirements of the EC Habitats Directive in the exercise of their functions'. This means that they must consider the above 3 tests when determining whether Planning Permission should be granted for developments likely to cause an offence under the Conservation Regulations. As a consequence, Planning Applications for such developments must demonstrate that the 3 tests will be passed.</p>
Nationally Protected	
Bats	<p>These animals receive full protection under the Wildlife and Countryside Act 1981 (as amended by the Countryside and Rights of Way Act 2000), which makes it illegal (subject to exceptions) to:</p> <ul style="list-style-type: none"> <li>• Intentionally kill, injure or take any such animal;</li> <li>• Intentionally or recklessly damage, destroy or obstruct any place used for shelter or protection by any such animal; and</li> </ul>

Species	Legal Status
	<ul style="list-style-type: none"> <li>Intentionally or recklessly disturb such animals while they occupy a place used for shelter or protection.</li> </ul>
Viviparous Lizard, Grass Snake, Slow-worm	These animals receive limited protection under The Wildlife and Countryside Act 1981 (as amended by the Countryside and Rights of Way Act 2000), which makes it illegal to intentionally kill or injure any such animal.
Nesting Birds (general)	All wild birds are protected under the Wildlife and Countryside Act 1981 (as amended by the Countryside and Rights of Way Act 2000), which makes it illegal (subject to exceptions) to: <ul style="list-style-type: none"> <li>Intentionally kill, injure or take any wild bird;</li> <li>Take, damage or destroy the nest (whilst being built or in use) or eggs of any wild bird.</li> </ul>
Wild Mammals	The Wild Mammals (Protection) Act 1996 makes it illegal to mutilate, kick, beat, nail, or otherwise impale, stab, burn, stone, drown, crush, drag or asphyxiate any wild mammal with intent to inflict unnecessary suffering.
Invasive Species	
None	-

4.1.2 Section 40 of the Natural Environment and Rural Communities Act 2006 (the NERC Act) places a legal duty on public bodies, including planning authorities, to ‘have regard’ to the conservation of biodiversity when carrying out their normal functions, which includes consideration of planning applications.

4.1.3 In compliance with Section 41 of the NERC Act, the Secretary of State has published a list of species and habitats considered to be of principal importance for conserving biodiversity in England under the UK Post-2010 Biodiversity Framework. This is known as the list of Habitats and Species of Principal Importance (HPI/SPI), of which there are 56 habitats and 943 species. The HPI/SPI list is used to guide planning authorities in implementing their duty under the NERC Act.

## 4.2 National Planning Policy

4.2.1 The NPPF set out the Government’s planning policies for England and how these are expected to be applied. At the heart of the NPPF is a presumption in favour of sustainable development. This presumption does not apply where development requiring Appropriate Assessment under the Birds or Habitats Directives is being considered, planned or determined.

4.2.2 The NPPF states that:

*‘When determining planning applications, local planning authorities should aim to conserve and enhance biodiversity by applying the following principles:*

- if significant harm resulting from a development cannot be avoided (through locating on an alternative site with less harmful impacts), adequately mitigated, or, as a last resort, compensated for, then planning permission should be refused;
- proposed development on land within or outside a Site of Special Scientific Interest (SSSI) likely to have an adverse effect on a SSSI (either individually or in combination with other developments) should not normally be permitted. Where an adverse effect on the site’s notified special interest features is likely, an exception should only be made where the benefits of the development, at this site, clearly outweigh both the impacts that it is likely to have on the features of the site that make it of special scientific interest and any broader impacts on the national network of SSSIs;
- development proposals where the primary objective is to conserve or enhance biodiversity should be permitted;

- opportunities to incorporate biodiversity in and around developments should be encouraged;
- planning permission should be refused for development resulting in the loss or deterioration of irreplaceable habitats, including ancient woodland and the loss of aged or veteran trees found outside ancient woodland, unless the need for, and benefits of, the development in that location clearly outweigh the loss; and
- the following wildlife sites should be given the same protection as European sites: potential Special Protection Areas (SPA) and possible Special Areas of Conservation (SAC); listed or proposed Ramsar sites; and sites identified, or required, as compensatory measures for adverse effects on European sites, potential SPAs, possible SACs, and listed or proposed Ramsar sites.'

4.2.3 Under the NPPF, the Planning Authority has a responsibility to promote the preservation, restoration and re-creation of priority habitats, ecological networks and the protection and recovery of priority species populations, linked to national and local targets, and identify suitable indicators for monitoring biodiversity in the plan.

4.2.4 Also under the NPPF the planning system should contribute to and enhance the natural and local environment by minimising impacts on biodiversity and providing net gains in biodiversity where possible, contributing to the Government's commitment to halt the overall decline in biodiversity, including by establishing coherent ecological networks that are more resilient to current and future pressures.

### 4.3 Local Planning Policy

4.3.1 The following policies of the adopted Barnsley Local Development Framework Core Strategy (Barnsley Council, 2011) are applicable:

- CSP 33 Green Infrastructure:  
"We will protect, maintain, enhance and create an integrated network of connected and multi-functional Green Infrastructure assets that:
  - i. provides attractive environments where people want to live, work, learn, play, visit and invest;
  - ii. meets the environmental, social and economic needs of communities across the borough and the wider City Regions;
  - iii. enhances the quality of life for present and future residents and visitors;
  - iv. helps to meet the challenge of climate change;
  - v. enhances biodiversity and landscape character;
  - vi. improves opportunities for recreation and tourism;
  - vii. respects local distinctiveness and historical and cultural heritage; and
  - viii. maximises potential economic and social benefits.

At a strategic level Barnsley's Green Infrastructure network includes the following corridors which are shown on the Green Infrastructure Diagram 5 [available on page 137 of the Barnsley Local Development Framework Core Strategy (Barnsley Council, 2011)]:

- i. River Dearne Valley Corridor;
- ii. River Dove Valley Corridor;
- iii. River Don Valley Corridor;
- iv. Dearne Valley Green Heart Corridor; and
- v. Historic Landscape Corridor.

The network of Green Infrastructure will be secured by protecting open space, creating new open spaces as part of new development, and by using developer contributions to create and improve Green Infrastructure.

We will produce a Green Infrastructure Strategy for Barnsley which will be informed by the Leeds City Region and South Yorkshire Green Infrastructure Strategies.”

- CSP 36 Biodiversity and Geodiversity:

“Development will be expected to conserve and enhance the biodiversity and geological features of the borough by:

- i. protecting and improving habitats, species, sites of ecological value and sites of geological value with particular regard to designated wildlife and geological sites of international, national and local significance, ancient woodland and species and habitats of principal importance identified in Section 74 of the Countryside and Rights of Way Act 2000 and in the Barnsley Biodiversity Action Plan;
- ii. maximising biodiversity and geodiversity opportunities in and around new developments; and
- iii. conserving and enhancing the form, local character and distinctiveness of the river corridors of the Dearne and Dove as natural floodplains and important strategic wildlife corridors.

Development which may harm a biodiversity or geological feature will not be permitted unless effective mitigation and/or compensatory measures can be ensured.”

- CSP 37 Landscape Character

“Development will be expected to retain and enhance the character and distinctiveness of the individual Landscape Character Area in which it is located (as set out in the Landscape Character Assessment of Barnsley Borough 2002).”

## 5 Discussions and Recommendations

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### 5.1 Overview of Potential Ecological Constraints

5.1.1 In the absence of mitigation, the following ecological features have been identified as potential constraints to the proposed works:

- Deciduous Woodland and Hedgerow HPI;
- Bats;
- Birds (general nesting);
- Common Reptiles; and
- England SPI/Local BAP & Notable Mammal and Invertebrate species.

### 5.2 Further Survey and Mitigation

5.2.1 For each potential constraint identified, all mitigation options provided follow the established mitigation hierarchy as set out in BS42020:2013 (BSI, 2013). This seeks as a preference to avoid impacts, then to mitigate unavoidable impacts, and as a last resort, to compensate for unavoidable residual impacts that remain after avoidance and mitigation measures. All recommended surveys and mitigation measures follow current best practice guidance as identified by CIEEM (CIEEM, 2014) and is proportionate to the level of impact identified and to the nature and scale of the proposed works. A clear and valid justification of methods has been provided where necessary.

#### *Deciduous Woodland and Hedgerow HPI*

5.2.2 **Avoidance:** Avoid all indirect impacts within root zones during the construction phase, through the installation of a buffer/protection fencing at appropriate distances in accordance with BS5837:2012.

5.2.3 **Mitigation:** None proposed.

5.2.4 **Compensation:** None proposed.

#### *Bats*

5.2.5 **Avoidance:** There will be no night lighting during construction activities, and a sensitive lighting strategy will be incorporated during operation of the site, to avoid indirect impacts to surrounding habitats and species (i.e. bats) that may utilise these features. If necessary, this can be subject to a planning condition or section 106 agreement.

5.2.6 **Mitigation:** To determine impacts and to inform the design of mitigation as necessary, a bat activity survey of the site will need to be undertaken in accordance with current guidance (Collins, 2016). This should include a single survey visit per season (spring/summer/autumn) in suitable weather conditions. Discussions can be held with the County Ecologist following completion of the first survey to determine whether subsequent surveys are still required (i.e. insignificant levels of bat activity recorded). A single automated/static detector will also need to be deployed on 5 consecutive nights per season. The undertaking of these surveys will be required prior to determination of planning.

5.2.7 **Compensation:** None proposed.

*Birds (general nesting)*

5.2.8 **Avoidance:** To avoid the disturbance of active nesting birds, any habitats will be cleared from September to February, outside of the bird nesting season. As timing constraints associated with other protected species (i.e. common reptiles) will also need to be taken into consideration, it may be necessary to clear suitable bird nesting habitat between March and August. In this instance, a suitably qualified Ecological Clerk of Works (ECoW) will survey habitat immediately prior to its removal. If an active nest is present, at least a 5 metre radius buffer area (or wider as appropriate and dependent upon the species identified) will be set out, or the vegetation retained until any young have fledged. To prevent contractors accidentally straying into the buffer area, these will be clearly marked out i.e. with high visibility fencing. If necessary, this can be subject to a planning condition and will not require any further survey prior to planning determination.

5.2.9 **Mitigation:** None proposed.

5.2.10 **Compensation:** None proposed.

*Common Reptiles*

5.2.11 **Avoidance:** None proposed (site clearance required).

5.2.12 **Mitigation:** To determine impacts and to inform the design of mitigation as necessary, a reptile survey will need to be undertaken of the site, in accordance with current guidance (Froglife, 1999). The survey should include any potential receptor site (i.e. adjacent habitat), so as to establish the current status of any reptile population within this and the associated carrying capacity. The undertaking of this survey will be required prior to determination of planning.

5.2.13 **Compensation:** None proposed.

*England SPI/Local BAP & Notable Mammal and Invertebrate species*

5.2.14 **Avoidance:** Any England SPI/Local BAP & Notable mammals or invertebrate species will be allowed to disperse into adjacent habitats during site clearance works, to avoid impacts to these species.

5.2.15 **Mitigation:** None proposed.

5.2.16 **Compensation:** None proposed.

5.2.17 To comply with guidance set out in BS42020:2013, a Construction Environment Management Plan (CEMP) will be produced prior to the commencement of construction activities, including site clearance works or archaeological investigations etc. A Landscape and Ecological Management Plan (LEMP) will then be produced prior to operation of the site to ensure the continued appropriate management and monitoring of ecological features. These documents should contain details on the mechanisms in place to secure mitigation works, and refer to the Construction (Design and Management) Regulations 2015 (CITB, 2016) where applicable. The production of both a CEMP and a LEMP can be requested within a planning condition.

### 5.3 Programme of Works

5.3.1 The recommended further surveys will be undertaken in accordance with the proposed programme of works (timings associated with mitigation will be fully detailed within any CEMP/LEMP document produced). The programme takes into account: seasonal survey constraints; the expected timetable of works (i.e. when site clearance needs to be undertaken, and when construction works are due to commence); and any other timing considerations, such as, planning application submission deadlines.

**Table 5.1: Programme of Works**

Recommendation	2016/17											
	J	F	M	A	M	J	J	A	S	O	N	D
Bat Activity Survey												
Reptile survey												

## 5.4 Opportunities for Biodiversity Enhancement

5.4.1 In accordance with national and local planning policy, opportunities for biodiversity enhancement (above and beyond those required to mitigate for the identified impacts) are set out below. Further details of these will be provided within any CEMP/LEMP document and if necessary, can be subject to a planning condition:

- Incorporation of native fruiting tree/shrub species of local provenance into the landscape design;
- Enhancement of the wider green infrastructure resource (i.e. improve hedgerow/woodland connectivity with disused railway and Edderthorpe Ings LWS);
- Enhancement of the River Dearne tributary (i.e. additional aquatic habitat);
- Installation of 2x 2F Schwegler bat boxes (or similar product) located upon boundary mature trees (building unlikely to be suitable to support), preferably south/south-west facing, at least 6ft from the ground and situated away from lighting/human disturbance; and
- Installation of 2x traditional wooden bird nest box (or similar product) located upon boundary mature trees, situated away from human disturbance.

## 6 Conclusion

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- 6.1.1 Pending the findings of the recommended bat and reptile surveys, and incorporation of all of the avoidance and mitigation measures outlined above, the proposed application should be able to avoid/mitigate any significant residual impacts to protected species and habitats.

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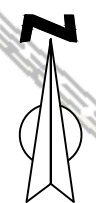
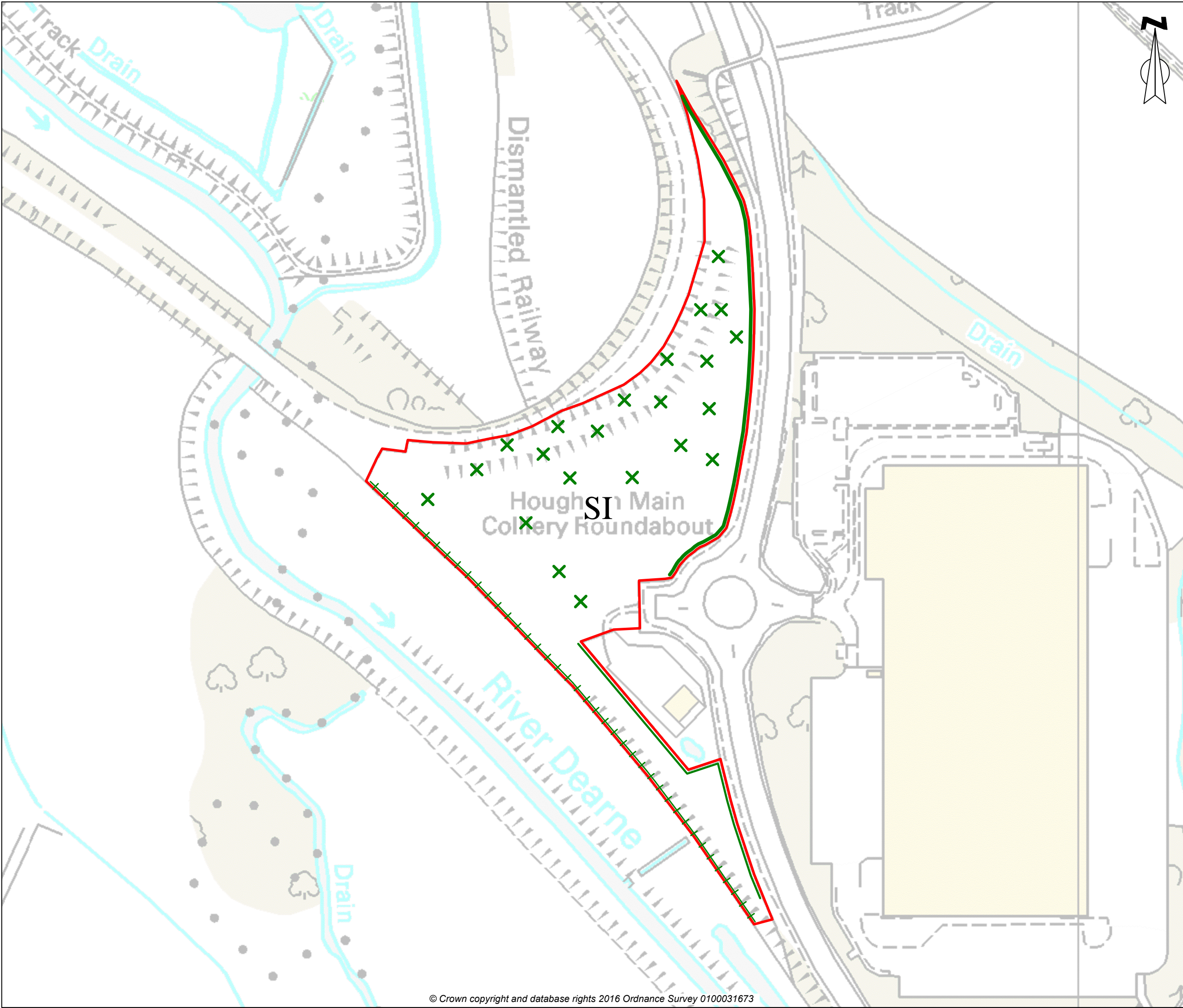
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## Drawing CRM.066.006.EC.D.001 – Phase I Habitat Map

---



- Key**
- Site Boundary
  - SI Poor Semi-Improved Grassland (B6)
  - X X X  
X X X Scattered Scrub (A2.2)
  - + + +  
+ + + Hedgerow with Trees (J2.3.2)
  - Intact Hedge (J2.1.2)



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CLIENT:  
**Peel Environmental Ltd**

SCALE: 1:2,500@A3      PROJECT REF: CRM.066.006

DRAWN: MG      CHECKED: KR      DATE: Aug 2016

PROJECT:  
**Houghton Main Colliery**

TITLE:  
**Phase 1 Habitat Map**

DRAWING NO:  
**CRM.066.006.EC.D.001**

## Appendix A- Proposed Site Layout

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## Appendix B- GCN eDNA Survey Methodology

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# 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 carnifex*) which is present at a few of locations. The primers and probe were also tested on tissue samples of marbled newt (*Triturus marmoratus*). None of these samples were amplified, confirming the suitability of the primer pair and probe for the great crested newt. The primers and probe also did not bind with the DNA of other UK native newts (smooth and palmate newt) which are in the genus *Lissotriton*.

## 2.5 Identification of risks of false positives and false negatives

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

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

- (i) Molecular assay design: mitigated in research and development phase of primer and probe design. Salt free primers should be used. The quality of the primer and of the PCR mix is assured by the standards.
- (ii) Laboratory quality control: mitigated by laboratory design and process control.
- (iii) Sampling design: mitigated by site selection procedures in field monitoring programmes.
- (iv) Uncertainty in the relationship between presence of target DNA and presence of viable target organisms: mitigated partially by research so far undertaken, and by future research increasing knowledge of great crested newt eDNA.

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

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

Risk factor	Mitigation
<b>Field-based false positives</b>	
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.
<b>Field-based false negatives</b>	
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
<b>Laboratory false positives</b>	
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.
<b>Laboratory false negatives</b>	
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 room<sup>1</sup>.
- **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.

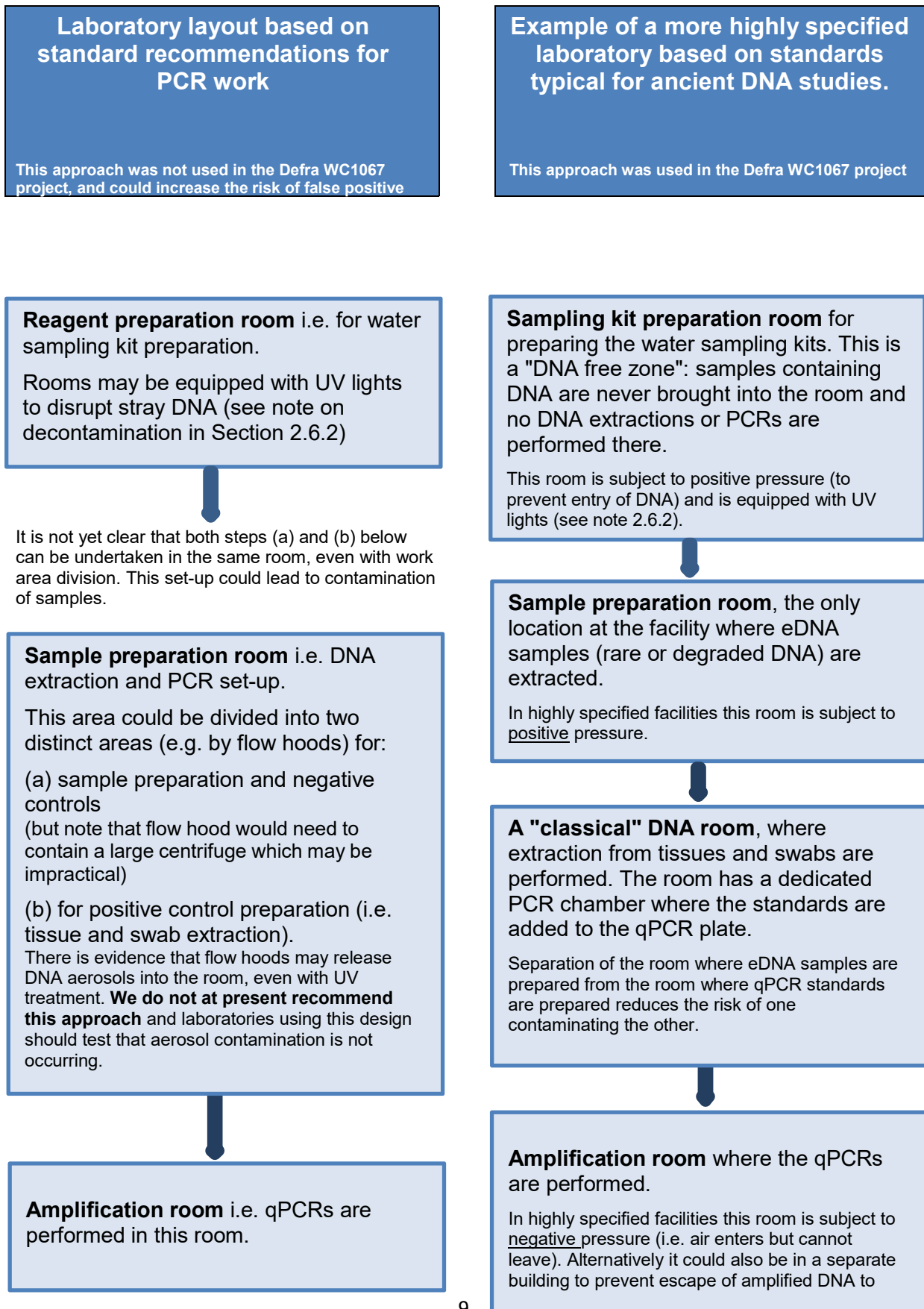
To reduce the risk of DNA contamination regular bleaching of the laboratory should be undertaken weekly. qPCR work should be undertaken inside a cabinet with UV light and in a room which is also lit by UV light outside the cabinet; to control aerosol DNA. Although UV lights are widely recommended for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

<sup>1</sup> 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.**



earlier preparation stages.

### 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 x g<sup>1</sup>, 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 6<sup>th</sup> 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

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

laboratory contamination. The extraction control is undertaken using an 11<sup>th</sup> 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  $\mu\text{L}^{-1}$  to  $10^{-4}$  ng  $\mu\text{L}^{-1}$  (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  $\mu\text{L}$  made up from:

- 3  $\mu\text{L}$  of template DNA
- 12.5  $\mu\text{L}$  of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®)
- 6.5  $\mu\text{L}$  of ddH<sub>2</sub>O
- 2  $\mu\text{L}$  of primer (1  $\mu\text{L}$  each of primer 10  $\mu\text{M}$  TCCBL and TCCBR)
- 1  $\mu\text{L}$  of probe (2.5  $\mu\text{M}$  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.

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<sup>2</sup>( $C_t$  =  $C_t$  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.

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

### Degradation control

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

## **Acknowledgements**

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

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 C- GCN eDNA Survey Results

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Technical Report  
Confidential

**Folio No:** D2066  
**Report No:** 1  
**Client:** Enzygo Ltd  
**Order No:** CRM2397  
**Attn:** Kirsty Rogers  
  
**Date:** 14<sup>th</sup> July 2016

## **TECHNICAL REPORT**

### **EXAMINATION OF ENVIRONMENTAL DNA**

### **IN POND WATER FOR THE DETECTION OF**

### **GREAT CRESTED NEWTS**

**T. Wood**



## SUMMARY

When Great Crested Newts (GCN) 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 (e-DNA) traces to confirm GCN inhabitation, or prove absence of presence.

The following samples were submitted for e-DNA analysis to the protocol stated in DEFRA WC1067 (Latest Amendments). Details on the sample submission form were used as the unique sample identity.

Date received at Laboratory: 4<sup>th</sup> July 2016  
 Date DNA extracted started: 4<sup>th</sup> July 2016  
 Date of analysis: 6<sup>th</sup> July 2016  
 Matters Affecting Results: None

## RESULTS

Laboratory Reference	Sample Detail	Co-Ordinates	Inhibition Check	Sample integrity	Result / Score
eDNA23287	CRM 066.006, Pond 3	SE4170406459	Acceptable	Acceptable	Negative 0/12
eDNA23288	CRM 066.006, Pond 2	SE4170406459	Acceptable	Acceptable	Negative 0/12
eDNA23289	CRM 066.006, Pond 1	SE4170406459	Acceptable	Acceptable	Negative 0/12

## METHODS AND INFORMATION.

In the above table, **NEGATIVE** means that GCN DNA 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 DNA 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.

Inhibition check refers to a laboratory evaluation of the sample tested by adding a known amount of an artificial gene to the sample and running the qPCR analysis in duplicate. Variation



in the point at which qPCR amplification starts indicates inhibition, which requires the sample to be diluted and retested.

Sample integrity 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. PCR inhibitors can cause false results. 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. In that case the result will be reported as inconclusive.

To generate the results all of the tubes from each pond are combined to produce one e-DNA 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.

A positive result may be a true positive but there is also a possibility that it is due to contamination of the sample from another pond, or improper sampling technique. Please ensure traditional surveys are performed on positive ponds and care is taken to avoid spreading GCN DNA from one site to another. The optimum accuracy of the method depends on careful sampling methodology alongside our forensic standards of laboratory analysis.

We provide training sheets and can offer formalised training in collection, even though certified training of collectors is not currently a Natural England requirement when sampling for GCN.

Kits are manufactured by Scientifics to strict quality procedures 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.

## **LABORATORY ANALYSIS**

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'.



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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 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 as follows:

<b>Primer</b>	<b>Sequence</b>	<b>Fragment</b>	<b>Gene</b>
TCCBL	CGTAAACTACGGCTGACTAGTACGAA	81	Cyt-b
TCCBR	CCGATGTGTATGTAGATGCAAACA	81	Cyt-b
<b>Probe</b>			
TCCB	CATCCACGCTAACGGAGCCTCGC	81	Cyt-b

Using this protocol, there should be no detection of closely related species. In the case of the great crested newt in the UK, the risk is mainly of detecting the Italian crested newt (*Triturus carnifex*) which is present at a few 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*.

Reported By: **Sam Humphrey**



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*D. H.*





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