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A survey of the River Dee for the Scarce Yellow Sally stonefly *Isogenus nubecula* in spring 2018

J. Davy-Bowker, Q. Mauvisseau, M. Hammett, M.
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Contents

1.	Crynodeb Gweithredol	1
2.	Executive Summary	2
3.	Introduction.....	3
3.1.	Status of <i>Isogenus nubecula</i>	3
3.2.	Purpose of 2018 survey.....	4
4.	Methods.....	4
4.1.	Field survey.....	4
4.2.	Laboratory analysis.....	5
4.3.	Captive rearing.....	6
4.4.	eDNA.....	6
5.	Results	7
5.1.	River levels.....	7
5.2.	<i>Isogenus nubecula</i> 2018 records.....	7
5.3.	Captive rearing.....	10
5.4.	eDNA.....	11
6.	Discussion	11
6.1.	River levels.....	11
6.2.	<i>Isogenus nubecula</i> 2018 records.....	12
6.3.	Captive rearing.....	12
6.4.	eDNA.....	13
6.5.	Habitat requirements.....	13
7.	Recommendations.....	14
8.	Acknowledgements.....	14
9.	References	14
10.	Appendix 1. <i>Isogenus nubecula</i> and sampling site photographs.....	16
11.	Appendix 2. 2018 survey notes.....	23
12.	Data Archive Appendix.....	29

List of Figures

Figure 1.	Daily river level in metres above sea level at Manley Hall gauging station over the four years 2015 to 2018.....	9
Figure 2.	Daily river level in metres above sea level at Manley Hall gauging station from the 1st of January 2018 to the 30th of April 2018	9
Figure 3.	Map of the River Dee and its major tributaries showing sites sampled and where <i>Isogenus nubecula</i> was found in in spring 2018	10

List of Tables

Table 1.	Sites planned for sampling in spring 2018 for <i>Isogenus nubecula</i>	5
Table 2.	Number of Perlodidae stoneflies found at sites sampled in spring 2018.....	8
Table 3.	Sites from which three replicate eDNA filters were obtained in spring 2018.....	Error!

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1. Crynodeb Gweithredol

Rhywoaeth pryf y cerrig sydd mewn perygl difrifol yw *Isogenus nubecula* a welir dim ond yn Afon Dyfrdwy yng ngorllewin Ewrop. Cafodd y rhywogaeth pryf y cerrig hon ei chanfod yn gyntaf yn Afon Dyfrdwy ym 1959 ac fe'i cofnodid o bryd i'w gilydd hyd at 1995. Roedd yn bresennol mewn naw o dri safle ar ddeg ym 1981 a 1982 dros ran 45km o hyd ar yr afon. Erbyn 1992, roedd ei hamrediad wedi lleihau'n sylweddol i ddau yn unig o'i chyn-safleoedd, er fe'i canfuwyd ar bum safle ym 1993. Ym 1995, dim ond un nymff a ganfuwyd ar un safle. Y safleoedd allweddol gydol y cyfnod hwn oedd Bangor Is-coed a Dolydd Pickhill. Methodd arolygon ym 1997, 1998, 1999, 2003, 2004, 2007, 2012 a 2015 â dod o hyd i *I. nubecula* a thybid bod y rhywogaeth wedi diflannu o Ynysoedd Prydain. Fodd bynnag, cafodd ei hailddarganfod yn Afon Dyfrdwy ym mis Mawrth 2017 wedi dwy flynedd o absenoldeb, wrth i nymffau gael eu canfod ym Mangor Is-coed, sef un o leoliadau hanesyddol y rhywogaeth.

Canfu'r arolwg presennol nymffau o *I. nubecula* ar bum safle ar ran 19.6km o hyd o Afon Dyfrdwy yng ngwanwyn 2018 – Erbistog (1 nymff), Owrtyn (1 nymff), Bangor Is-coed (16 o nymffau), Dolydd Pickhill (69 o nymffau) a Wern (3 nymff). Ni chanfuwyd dim nymffau yn Afon Ceiriog nac yn Afon Alun, sy'n awgrymu bod *I. nubecula* yn bodoli dim ond ym mhrif wely Afon Dyfrdwy. Felly, mae *I. nubecula* yn parhau'n agored iawn i ddiflannu yn sgil digwyddiadau llygredd neu golli cynefinoedd.

Bu magu nymffau a gasglwyd mewn caethiwed yn dangos y bu iddynt godi o'r dŵr cryn amser cyn dod i'r amlwg ac mae'n debyg y digwyddodd hynny dros nos yn bennaf, gyda mwyafrif yr oedolion yn cael eu gweld yn gyntaf yn gynnar y bore canlynol. Uchafswm oes oedolion mewn caethiwed oedd oddeutu un mis (er yr oedd yn aml yn llai).

Cafwyd tri chopi o hidlenni eDNA o bob un o'r deuddeg o safleoedd yr ymwelwyd â nhw: deg o Afon Dyfrdwy, un o Afon Ceiriog ac un o Afon Alun. Canlyniad dadansoddiad DNA o'r samplau eDNA, a ddadansoddwyd gan dîm Prifysgol Derby gan ddefnyddio peiriant qPCR (adwaith cadwynol polymerasau meintiol), oedd negatiff. Gallai hyn fod yn sgil tyrfedd uchel y dŵr.

Argymhellir gwaith pellach er mwyn:

- Ymgymryd ag arolwg pellach i ddarganfod dosbarthiad a statws cyfredol *I. nubecula* yn Afon Dyfrdwy;
- Ymgymryd ag arolygon cic-samplu cyfnodol i gael gwell dealltwriaeth o'r newidiadau yng nghynefin y rhywogaeth hon a'i dewisiadau o ran cynefin ac amgylchedd;
- Archwilio'r posibilrwydd o fagu *I. nubecula* mewn caethiwed a'i ailgyflwyno i'w gyn-safleoedd a safleoedd addas eraill gerllaw;
- Datblygu a phrofi technoleg DNA amgylcheddol i ganiatáu darganfod eDNA *I. nubecula* o samplau dŵr;
- Cynnal arolwg o gynefinoedd a nymffau yn yr haf (llif isel) i ganfod dewisiadau penodol o ran cynefinoedd, llywio'r broses o adfer afonydd, a galluogi cyfathrebiadau o ran rheolaeth briodol o afonydd.

2. Executive Summary

The Scarce Yellow Sally *Isogenus nubecula*, is a critically endangered stonefly found only in the River Dee in western Europe. First found in the Dee in 1959, the stonefly was recorded episodically up until 1995. It was present at nine of thirteen sites in 1981 and 1982 over a 45km stretch of the river. By 1992, its range had reduced significantly to just two of its former sites, although it was found at five sites in 1993. In 1995, a single nymph was found at one site. Key sites throughout this period were Bangor-on-Dee and Pickhill Meadows. Surveys in 1997, 1998, 1999, 2003, 2004, 2007, 2012 and 2015 failed to locate *I. nubecula* and it was presumed to be extinct in the British Isles. However, it was rediscovered in the River Dee in March 2017 following a 2-year period of absence, with nymphs found at Bangor-on-Dee, one of its historical locations.

The current survey found nymphs of *I. nubecula* at five sites on a 19.6km stretch of the River Dee in spring 2018 – Erbistock (1 nymph), Overton Bridge (1 nymph), Bangor-on-Dee (16 nymphs), Pickhill Meadows (69 nymphs) and Wern (3 nymphs). No nymphs were found in the River Ceiriog or River Alyn, suggesting that *I. nubecula* is restricted to the main channel of the Dee. As such, *I. nubecula* remains highly vulnerable to loss from pollution incidents or habitat loss.

Captive rearing of collected nymphs showed that nymphs climbed out of the water some time before emerging and that emergence seemed to occur predominantly overnight, with most adults first seen early the following morning. Adult maximum longevity in captive conditions was about one month (although often less).

Three replicate eDNA filters were obtained from all twelve sites visited, ten from the River Dee and one each from the River Ceiriog and River Alyn. The eDNA samples, analysed by the University of Derby team using a qPCR (quantitative polymerase chain reaction) machine, all proved negative for the DNA analysis. This may have been due to the high turbidity of the water.

Further work is recommended to:

- Undertake further survey work to determine the current status and distribution of *I. nubecula* in the River Dee;
- Undertake periodic kick sampling surveys to better understand changes in the range of this species and its habitat and environmental preferences;
- Explore the potential for captive breeding and reintroduction of *I. nubecula* to its former sites and other suitable sites nearby;
- Develop and test environmental DNA technology to permit detection of *I. nubecula* eDNA from water samples;
- Perform a summer (low flow) habitat and nymph survey to identify specific habitat preferences, guide river restoration, and enable the communication of appropriate river management.

3. Introduction

3.1. Status of *Isogenus nubecula*

The Scarce Yellow Sally *Isogenus nubecula* (Newman, 1833) is a member of the family Perlodidae, part of the insect order Plecoptera (stoneflies). *I. nubecula* is a very rare species and has recently been assigned the international threat category 'Critically Endangered' in a recent review by Macadam (2015). *I. nubecula* was first recorded in Great Britain in 1959 from the River Dee at Bangor-on-Dee in North Wales (Hynes, 1963). This site has a substratum consists of large gravel and cobbles, much of which is unstable due to high water velocity, a stone road bridge and a large within-channel island (Figure 11).

A detailed account of the UK records of *I. nubecula* between its discovery in 1959 and 2003 is provided by Davy-Bowker (2003). In summary, the presence of *I. nubecula* in the River Dee was confirmed in the surveys carried out in 1981 and 1982 by Mills and Andrew (1984) where its maximum range was recorded at nine of thirteen sites surveyed over a 45km stretch of the river. In 1992, the range of *I. nubecula* had reduced significantly, to just two of its former sites (Bowker, 1993). However, it was found at five sites in 1993 (Bowker, 1995), though this reduced again two years later and by 1995 only one nymph was found at one site (Tanner, 1997). Despite repeated surveys conducted in 1997 (Tanner, 1997), 1998 (Millband, 1998), 1999 (Millband, 1999), 2003 and 2004 (Davy-Bowker, 2003; Hammett & Wallace, 2005), 2007 (Davy-Bowker *et al.*, 2007) and unpublished surveys carried out by John Davy-Bowker in 2012 and 2015, no further specimens of *I. nubecula* were found on the River Dee. *I. nubecula* was therefore presumed to be extinct from the River Dee and hence from the British Isles.

Isogenus nubecula is rare throughout Europe and has been lost from many countries (Davy-Bowker, 2003). Derka *et al.* (2002) described *I. nubecula* as a species originally recorded from many localities in western and central Europe, and Zwick (1992) noted its widespread disappearance in the second half of the 20th century. In central and eastern Europe, Claessens (1981) found no records since 1936 from Austria, Belgium, Switzerland and the Netherlands, and Landa *et al.* (1997) considered that *I. nubecula* had probably also become extinct in the Czech Republic and Slovenia.

Surviving European populations are likely to be few in number and occur mainly in eastern Europe in the Lafnitz and Rába rivers in south-eastern Austria and western Hungary (Graf & Kovács, 2002; Kovács & Ambrus, 2001), the River Tisza in Hungary (Juhász *et al.*, 1998; Kovács & Ambrus, 2001), the River Jelesna in Slovakia where a single nymph was found in 1998 (Derka *et al.*, 2002), Northrhine-Westfalia in Germany (Graf *et al.*, 2009) and in central Sweden (Hoffsten, 2003). *I. nubecula* may potentially also occur in Finland, Estonia, Latvia and Lithuania.

On 12th March 2017, John Davy-Bowker made a further visit to the River Dee at Bangor-on-Dee and was extremely fortunate to rediscover the species (Davy-Bowker *et al.*, 2018). This rediscovery came after a 22-year period of absence despite numerous surveys since its previous record in 1995. A follow-up survey was also performed by Mike Hammett on 15th March 2017 and the presence of *I. nubecula* was confirmed.

Isogenus nubecula is one of the rarest stoneflies in the UK and Europe and its re-discovery is of international significance. The River Dee is the only location in Western Europe where this species can still be found. Only fourteen individuals were caught in March 2017 and the need for conservation and increased survey effort is therefore of paramount importance.

Following the 2017 rediscovery, Natural Resources Wales commissioned a resurvey in spring 2018 of sites where *I. nubecula* had been historically found on the River Dee so that its current distribution could be better understood. This report describes that survey and presents the results.

3.2. Purpose of 2018 survey

This survey had the principle aim of updating knowledge on the spring 2018 distribution of *I. nubecula* in the River Dee and its major tributaries by surveying all sites where it had formerly been recorded. Additional goals (if possible) included:

- Submission of all UK records of *I. nubecula* to the National Biodiversity Network;
- Lodging of voucher specimen(s) with the Natural History Museum (ideally ethanol preserved to permit subsequent DNA recovery);
- Captive rearing of *I. nubecula* to adult if possible to confirm identity and to gather further knowledge about the species for potential future captive breeding;
- Obtaining temperature logger data from the River Dee to better understand the thermal tolerance of *I. nubecula*;
- eDNA survey of the River Dee and its major tributaries to see if environmental DNA from *I. nubecula* could be recovered*;
- A brief scientific paper to place a note of any new 2018 records in the scientific literature.

*eDNA note: The River Dee is a large and difficult to sample river, especially in the early spring when flows are typically high. Unfortunately, this is also the best time to collect *I. nubecula* nymphs as they are large at this time of year and therefore easier to find and identify. As well as kick sampling surveys, a further way to increase survey effort is by eDNA (environmental DNA). One of the specimens of *I. nubecula* found by John Davy-Bowker on 12th March 2017 was therefore sent to collaborators Quentin Mauvisseau, Michael Sweet and Chris Troth at the University of Derby and work was commenced on developing an eDNA primer by the Derby team so that in future *I. nubecula* eDNA could be detected from water samples (Davy-Bowker *et al.*, 2018). A cytochrome oxidase subunit I (COI) DNA sequence of *I. nubecula* has subsequently been uploaded to GenBank making it accessible for other researchers and work on developing and testing an eDNA primer for *I. nubecula* is ongoing.

4. Methods

4.1. Field survey

All sites where *I. nubecula* had formerly been recorded on the River Dee were resampled in spring 2018. A list of target sampling sites is given in Table 1. Sites were surveyed in early 2018 (March and April). A balance was struck between high winter river levels (forcing delays to the survey) and the need to sample before mid-late April when most *I. nubecula* nymphs can be expected to have emerged as adults and hence left the river. Regular reference was made to both the Met Office website www.naturalresourceswales.gov.uk

for current and long-range weather forecasts and to online data from the Manley Hall flow gauging station on the River Dee located just to the south of Manley House, near Erbistock (SJ 34840 41474).

Table 1. Sites planned for sampling in spring 2018 for *Isogenus nubecula*.

Site	Grid reference
River Dee at Froncysyllte	SJ 270 420
River Dee at Newbridge	SJ 288 416
River Dee at Coed Leodd	SJ 319 394
River Ceiriog u/s River Dee	SJ 316 392
River Dee at Erbistock	SJ 354 412
River Dee at Overton Bridge	SJ 355 427
River Dee at Bangor-on-Dee	SJ 387 454
River Dee at Pickhill Meadows	SJ 397 463
River Dee d/s River Clywedog	SJ 412 472
River Dee at Wern	SJ 41 3479
River Alyn u/s River Dee	SJ 395 561
River Dee downstream of the River Alyn	SJ 400 559

From past experience and discussions with Mike Hammett, a Manley Hall water level of 0.75m was taken to represent a river level at which it was likely that kick-sampling could be carried out at all sites. In the final event, river levels were high at the time fieldwork was scheduled to take place so that whilst a single fieldtrip was originally planned by John Davy-Bowker from Dorset to North Wales, high river levels created significant difficulties on that first fieldtrip and a second fieldtrip had to be undertaken to complete the survey. The first fieldtrip was carried out between 9th and 16th March 2018 and the second between 31st March and 1st April 2018.

Kick samples were taken at each site using a standard FBA pattern kick-sampling net with a 1mm mesh. Sampling duration varied between sites but was much longer than the normal 3-minute kick sample. Samples were picked for Perlodidae species in a white bottom tray at the river bank. The total time spent kick sampling and picking out Perlodidae ranged between 45 minutes and 2 hours, the duration being determined by the extent of accessible river and the success in finding Perlodidae specimens. Perlodidae specimens were either preserved at the river bank in 99% ethanol in plastic tubes or kept alive with water aeration and cooling. Perlodidae were subsequently transported back to the River Laboratory in Dorset. A site photograph was taken on each sampling occasion (Appendix 1) and the Manley Hall river level was also noted for future reference.

4.2. Laboratory analysis

Perlodidae specimens were identified using a low-power binocular microscope and cold light source in the laboratory (preserved specimens) or retained for adult rearing (followed by microscope identification). All Perlodidae nymphs (including those tentatively identified in the field and any adults reared) were identified to species level using the key by Hynes (1977) and with reference to John Davy-Bowker's personal reference collection of stoneflies that includes fresh reference specimens of *I. nubecula* obtained from the River Dee by the author in 2017 and examples of the other three non-extinct UK Perlodidae stonefly species - *Perlodes mortoni*, *Isoperla grammatica* and *Diura bicaudata*.

4.3. Captive rearing

Captive rearing of live *I. nubecula* nymphs was attempted in Dorset. This served two purposes. Firstly, adults can be used to help confirm species identity. Secondly, captive rearing creates possibilities to learn about the ecology and thermal tolerances of *I. nubecula* nymphs, the habits of the adults including drinking, feeding, drumming and mating behaviours, and characteristics of the eggs including morphology, survivability and time to hatching. If eggs could be hatched, it would also give a unique opportunity to learn about the juvenile nymphs which are very poorly known.

All live candidate *I. nubecula* nymphs from Bangor-on-Dee (collected on 9th March 2018) and from Pickhill Meadows (collected on 9th and 12th March and on 1st April 2018) were pooled as a single 'Bangor to Pickhill' sample to maximise the number of individuals and hence the possibility of success with captive rearing and adult mating.

Nymphs were reared in a cool box with regular, typically twice daily, ice pack changes to prevent water temperatures rising excessively. Within the cool box, rearing was carried out in plastic containers with River Dee water oxygenated using air pumps. Containers were free from mineral substrate but contained aquatic vegetation and debris as well as saturated twigs and tree bark from the sampling sites to provide habitat.

Large *I. nubecula* nymphs are carnivores and freshly collected live invertebrates (excluding Perlodidae stoneflies) were added at approximately weekly intervals as food. These mainly comprised Simuliidae fly larvae which appear to be easily predated by mature carnivorous stonefly nymphs and were available in large numbers from various local rivers.

When the nymphs were judged to be close to emergence (evidenced by a darkening of the wing pads) the containers were replaced with ones fully lined with mesh on their vertical side walls and extending into the air space above the water. This provided a means for nymphs to climb out of the water freely as well as giving adults a place to rest. Emerged and hardened adults were moved to containers without water and allowed to mix in the hope they might mate. A piece of wet moss was provided so that adults could drink. Regular checks were made for drumming and mating behaviour and to check for the presence of egg masses either attached to the females or deposited in the containers.

4.4. eDNA

As described in the Introduction, a COI DNA sequence of *I. nubecula* has been isolated from a 2017 specimen by the team at the University of Derby and work is ongoing to develop and test an eDNA primer for *I. nubecula*. The 2018 fieldwork was an opportunity to develop this work further by collecting a comprehensive set of eDNA samples matched to invertebrate kick samples across the full known historical range of *I. nubecula*.

Three replicate sets of water samples were taken at each site, filtered on site, and the filters refrigerated and retained for subsequent analysis to recover *I. nubecula* DNA. The number of syringes that could be pushed through each filter was recorded – this acted as a surrogate measurement of water turbidity. All equipment was kept contaminant-free by the use of disposable rubber gloves and eDNA samples were

collected prior to kick sampling to reduce sediment disturbance and the risk of contamination of eDNA samples between sites. To build knowledge about the persistence and recoverability of eDNA from different watercourses, a number of basic water chemistry parameters were also recorded using a handheld meter.

The persistence of eDNA on filters is limited. To prevent unacceptable degradation of DNA, all refrigerated filters were frozen on return from each day of fieldwork. Filters were transported to the University of Derby following the second fieldwork session for subsequent analysis.

5. Results

5.1. River levels

The River Dee is a large lowland river with an average discharge of 31.03 m³/s at the Manley Hall gauging station (Wikipedia.org). River levels fluctuate greatly and the Manley Hall water level of 0.75m (representing a river level at which it is likely that kick sampling can be carried out) is often exceeded (Figure 1). The occasions when river levels were below 0.75m during the best sampling period for *I. nubecula* (February and March) from 2015 to 2018 were interrupted by numerous periods when river levels were too high. This makes River Dee kick sampling inherently difficult and fieldwork planning problematic.

The fieldwork trip to the River Dee was scheduled to take place between 9th and 16th March 2018. This fieldwork went ahead but shortly after starting the river level rose (Figure 2). Sampling continued but some sites were too deep to sample on this visit. When fieldwork ceased, river levels continued to rise and didn't fall back below 0.75m until about 25th March.

A second attempt to complete the sampling was planned for 31st March and 1st April 2018. As with the previous visit, shortly after commencing fieldwork the river level began to rise, again exceeding 0.75m (Figure 2). Most of the missing samples were collected during this second visit. A few days after the second fieldwork visit was completed, river levels rose even further, peaking at approximately 1.5m and staying above 0.75 until approximately 15th April.

Despite these challenges, most sites were kick-sampled. Two exceptions were the River Dee downstream of the River Clywedog (Figure 13) and the River Dee downstream of the River Alyn (Figure 16). The Figures give an indication of the size of the River Dee is at these locations and how difficult it is to obtain kick samples under high flow conditions. Detailed field notes made during the sampling are given in Appendix 2.

5.2. *Isogenus nubecula* 2018 records

Isogenus nubecula was found at five sites in spring 2018, namely the River Dee at Erbistock, Overton Bridge, Bangor-on-Dee, Pickhill Meadows and Wern (Table 2), a distance of 19.6km.

At the upstream River Dee sites, Erbistock and Overton Bridge, only a single specimen of *I. nubecula* was found. At the downstream site, Wern, only three specimens were found. The maximum number of specimens of *I. nubecula* were

found at Bangor-on-Dee (16 specimens) and Pickhill Meadows (30 specimens). Two subsequent revisits to Pickhill Meadows to gather further specimens for captive rearing gave a further seven *I. nubecula* on 12th March, and a further 32 *I. nubecula* on 1st April. This gave a total *I. nubecula* count at Pickhill Meadows across three site visits of 69 specimens.

Throughout all previous surveys, Bangor-on-Dee and Pickhill Meadows have always held the strongest populations of *I. nubecula* and this pattern was seen again in 2018 (Figure 3). In this 2018 survey, Pickhill Meadows had a larger population than Bangor-on-Dee. Had high river levels not confined sampling at Pickhill Meadows to a narrow strip down one margin, it is possible that the numbers of *I. nubecula* at Pickhill Meadows could have been even larger.

Perlodes mortoni and *Isoperla grammatica* were found at all of the River Dee sites sampled. *I. grammatica* was the most numerous Perlodidae species, followed by *P. mortoni*. Both these species were also found on the River Ceiriog while no Perlodidae species were found on the River Alyn. The River Clywedog was not sampled because its water quality was regarded as too poor.

Table 2. Number of Perlodidae stoneflies found at sites sampled in spring 2018 (*stoneflies identified by Mike Hammett; †collected only *I. nubecula* for captive rearing; ‡collected only large Perlodidae for captive rearing).

Site	Grid reference	Date found	<i>Isoperla grammatica</i>	<i>Perlodes mortoni</i>	<i>Isogenus nubecula</i>
River Dee at Froncysyllte	SJ 26760 42029	31 March 2018	44	6	0
River Dee at Newbridge	SJ 28778 41666	15 March 2018	50	24	0
River Dee at Coed Leodd	SJ 31349 39977	31 March 2018	21	8	0
River Ceiriog u/s River Dee	SJ 31593 39155	11 March 2018	14	6	0
River Dee at Erbistock	SJ 35423 41242	14 March 2018	32	16	1
River Dee at Overton Bridge	SJ 35453 42744	14 March 2018	22	1	1
River Dee at Bangor-on-Dee	SJ 38773 45480	9 March 2018	14*	19	16
River Dee, Pickhill Meadows	SJ 39725 46309	9 March 2018	23	24	30
River Dee, Pickhill Meadows	SJ 39725 46309	12 March 2018	-†	-†	7
River Dee, Pickhill Meadows	SJ 39725 46309	1 April 2018	-†	16	32
River Dee d/s River Clywedog	SJ 41320 47321	Could not sample	n/a	n/a	n/a
River Dee at Wern	SJ 41331 47665	1 April 2018	14	4	3
River Alyn u/s River Dee	SJ 38236 56320	11 March 2018	0	0	0
River Dee d/s River Alyn	SJ 40049 55715	Could not sample	n/a	n/a	n/a

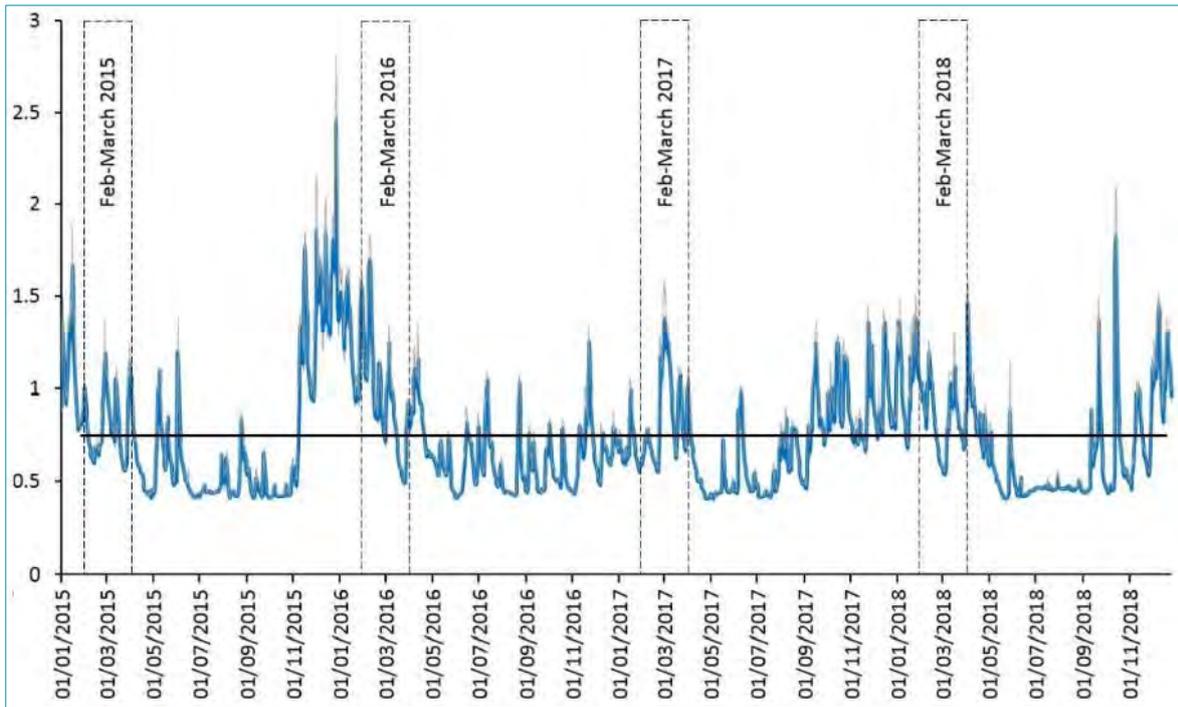


Figure 1. Daily river level in metres above sea level at Manley Hall gauging station over the four years 2015 to 2018. Maximum depth for kick sampling (0.75m) is indicated by the horizontal line. The ideal spring (February-March) survey period is indicated by dashed boxes.

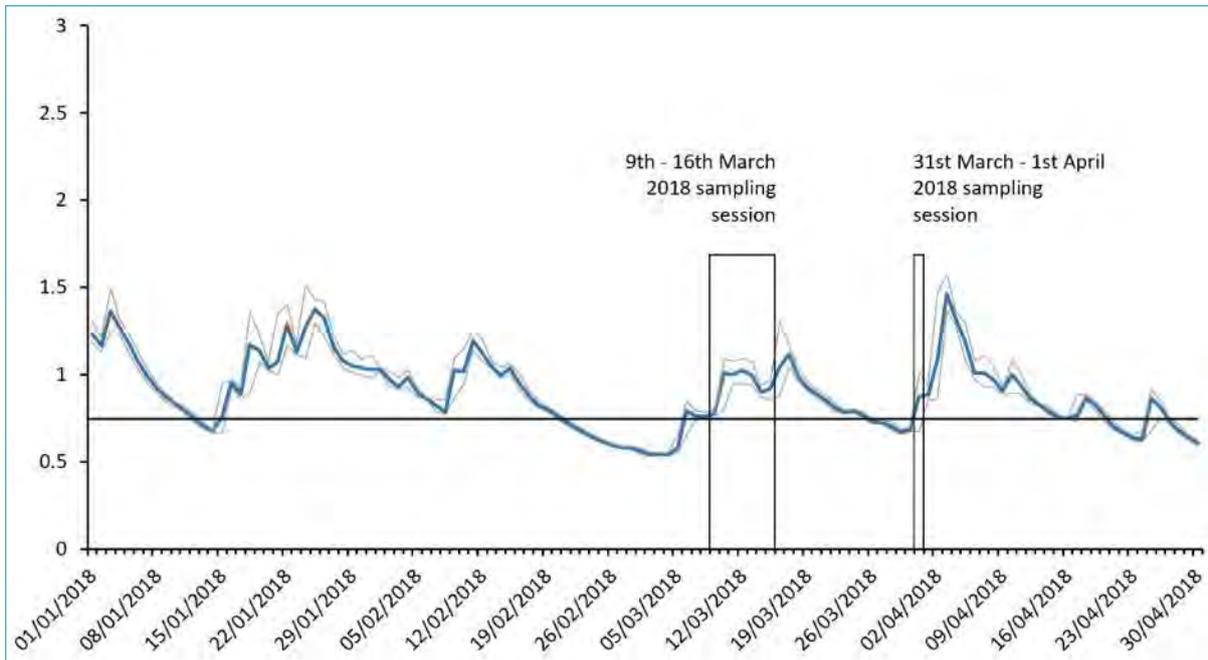


Figure 2. Daily river level in metres above sea level at Manley Hall gauging station from the 1st of January 2018 to the 30th of April 2018. Maximum depth for kick sampling (0.75m) is indicated by the horizontal line. The two spring 2018 survey sessions are indicated by the solid boxes.



Figure 3. Map of the River Dee and its major tributaries showing sites sampled and where *Isogenus nubecula* was found in in spring 2018. Number found in parentheses (Pickhill Meadows had three site visits).

5.3. Captive rearing

As with previous rearing work done by both John Davy-Bowker and Mike Hammett, rearing mature stonefly nymphs to adult is relatively straightforward and a small number of *I. nubecula* nymphs were successfully reared to adult from the 2018 River Dee samples. Despite this success, it is important to realise that there were a low number of starting specimens and therefore a low number of adults emerged. This resulted in difficulty obtaining and maintaining adults of both sexes with adequate fitness to mate. While at least one mating coupling event was seen, no eggs could be obtained.

Adult rearing of 2018 nymphs did permit some useful observations about the ecology of *I. nubecula* to be made, specifically, *I. nubecula* nymphs were observed to have climbed out of the water onto the side mesh of the rearing container some time before emerging. A second useful observation was that emergence seemed to occur predominantly overnight, with most adults first seen early the following morning.

Thirdly, adult maximum longevity in these captive conditions was about one month (although often less).

5.4. eDNA

Three replicate eDNA filters were obtained from all twelve sites visited, ten from the River Dee and one each from the Rivers Ceiriog and Alyn (Table 3).

Table 3. Sites from which three replicate eDNA filters were obtained in spring 2018.

Site	Grid reference	Date sampled
River Dee at Froncysyllte	SJ 26760 42029	31 March 2018
River Dee at Newbridge	SJ 28778 41666	15 March 2018
River Dee at Coed Leodd	SJ 31349 39977	31 March 2018
River Ceiriog u/s River Dee	SJ 31593 39155	11 March 2018
River Dee at Erbistock	SJ 35423 41242	14 March 2018
River Dee at Overton Bridge	SJ 35453 42744	14 March 2018
River Dee at Bangor-on-Dee	SJ 38773 45480	9 March 2018
River Dee at Pickhill Meadows	SJ 39725 46309	9 March 2018
River Dee d/s River Clywedog	SJ 41320 47321	1 April 2018
River Dee at Wern	SJ 41331 47665	1 April 2018
River Alyn u/s River Dee	SJ 38236 56320	11 March 2018
River Dee d/s River Alyn	SJ 40049 55715	15 March 2018

The eDNA samples were analysed by the University of Derby team using a qPCR (quantitative polymerase chain reaction) machine at Derby. All results were negative for the DNA analysis. This was likely due to the high turbidity of the water. A second analysis by the Derby team with a ddPCR (droplet *digital* polymerase chain reaction) machine in Belgium is now planned. This second analysis is anticipated to give better results as the method will not be impacted by the inhibiting effects of high turbidity (Quentin Mauvisseau, pers. comm.).

6. Discussion

6.1. River levels

The high water level of the River Dee presented challenges for the 2018 *I. nubecula* survey necessitating two separate field trips to the River Dee to complete the sampling. Despite these difficulties, almost all of the sites were sampled. The use of a dry suit was a key factor in being able to take kick samples with River Dee water levels being much higher than the normally accepted maximum level of 0.75m. The two sites that could not be kick-sampled were the River Dee downstream of the River Clywedog and the River Dee downstream of the River Alyn. Both of these sites are very difficult to kick-sample even at low river levels.

For future surveys, River Dee water levels will remain an important factor governing sampling success. There is an ongoing need to consider river levels versus the competing need to sample early in the spring to catch *I. nubecula* nymphs when they are large and therefore more easily found and easier to identify. The benchmark 0.75m at the Manley Hall gauging station remains a sensible measure of the ability to take kick samples. Regular reference to the Manley Hall gauge, long range weather forecasts, and in some years an awareness of the possibility of flow peaks arising from mountain snow melt at the top of the catchment, are all factors that need to be considered when undertaking *I. nubecula* kick sampling surveys.

6.2. *Isogenus nubecula* 2018 records

The new 2018 records of *I. nubecula* from this survey are both useful and encouraging. After a 22-year absence since *I. nubecula* was previously recorded in 1995, and following its rediscovery at the Bangor-on-Dee site in March 2017 (Davy-Bowker *et al.*, 2018), this survey has now updated our understanding of the distribution of this species to five sites on the River Dee, two of which (Bangor-on-Dee and Pickhill Meadows) appear to support strong populations.

Isogenus nubecula still only occurs on one river and none of the major tributaries appears to have populations. Despite an increase in the number of sites known from one to five, *I. nubecula* remains highly vulnerable to loss from pollution incidents or habitat loss.

The question of why *I. nubecula* was not detected for 22 years despite repeated sampling effort is difficult to answer. It must surely be the case that *I. nubecula* has been present at least somewhere on the River Dee river system during this intervening period. We do not know if *I. nubecula* remained present but undetected at its stronghold Bangor-on-Dee and Pickhill Meadows sites, or if it was absent from these sites yet persisted somewhere else outside the study area from where it has recolonised. It is possible that eDNA may provide a way to address this question in the future.

6.3. Captive rearing

Not being able to breed *I. nubecula* in captivity in 2018 was not surprising given the low number of starting individuals. Nevertheless, rearing mature nymphs to adults has enabled useful information on their ecology to be obtained that would have been difficult to gather from field surveys. Observations have been made of mature nymphs climbing out of the water and exploring the terrestrial environment just prior to emergence, a tendency for emergence to occur overnight, and some information on adult longevity in captivity has been obtained. Newly emerged adults are soft-bodied and pale in colour and the observation that emergence tends to occur at night could give two benefits in the wild. Firstly, it might reduce the risk of dehydration during the day, and secondly it might reduce bird predation. The observation of a roughly one month maximum adult lifespan in captivity underlines the need to try to maximise adult fitness in captive rearing so survival can be maintained whilst awaiting the emergence of mates.

Since the 2018 field survey and its associated captive rearing work, John Davy-Bowker has developed a new stonefly rearing system to improve upon the use of cool boxes and ice packs which is labour intensive, time-consuming and only permits

crude control of water temperature. John now has a commercial bottle cooling refrigerator converted for the purposes of stonefly rearing. This incorporates an Arduino microprocessor to control temperature more closely, thereby creating chosen thermal regimes that can more closely mimic the characteristics of rivers. Programmability enables monthly changes in water temperature to be reproduced as well as day-night thermal cycles. Illumination is also microprocessor-controlled to mimic a realistic day-night cycle. This system will be tested with commonly occurring species such as *Isoperla grammatica* with a view to future use with *I. nubecula*. The 2018 *I. nubecula* rearing work has provided useful information to help in the ongoing process of refining these rearing techniques. The ultimate hope is to be able to rear *I. nubecula* through complete life cycles in captivity for reintroduction to sites from which it has been lost.

6.4. eDNA

eDNA water sample filters were successfully obtained from all twelve sampling sites. While the initial qPCR runs to extract DNA have not yielded results, it is hoped that the planned ddPCR work will enable DNA to be extracted.

eDNA sampling has some unique potential benefits. These should make it a useful adjunct to kick sampling. Firstly, it is relatively unaffected by differences in sampling site water depth and whole channel accessibility. This should help to avoid some of the potential variability that can arise in kick sampling results which are more affected by these issues. Secondly it could give higher detection rates where populations are small. This could help address the question of where *I. nubecula* survives when it cannot be found by kick sampling and thereby clarify the natural range of this species. Thirdly, eDNA studies increase our understanding of phylogenetic relationships between different stonefly populations and species. This could help to determine the degree of genetic difference that may occur between UK and mainland European *I. nubecula* populations.

eDNA approaches could also be applied to the detection of other rare or threatened native freshwater species. Within the UK native stonefly and mayfly fauna, there are several species that could also benefit from the development of eDNA primers and the use of eDNA field surveys.

6.5. Habitat requirements

The absence of *I. nubecula* from other large lowland rivers in the United Kingdom, and from major tributaries of the River Dee itself, suggests that something is unique about the Bangor-on-Dee and Pickhill Meadows sites. However, it is not obvious what habitat feature or combination of features makes these sites suitable when so many others are not.

Bankside and within-channel habitat surveys (during low summer flows when the river is accessible) matched to quantitative juvenile nymph sampling could reveal the particular habitat features that favour *I. nubecula*. River restoration could be used to promote those habitat features and thereby increase the likelihood of the long-term survival of *I. nubecula*. River management could also benefit from the communication of the importance of these habitat features to stakeholders.

7. Recommendations

Now that *I. nubecula* has been rediscovered in the UK and its known distribution on the River Dee extended from one site in 2017 to five sites in 2018, it is important to build upon this success by continuing to monitor this rare species by kick sampling, further developing captive rearing techniques and continuing to conduct research on eDNA.

Further work is recommended to:

- Undertake further survey work to determine the current status and distribution of *I. nubecula* in the River Dee;
- Undertake periodic kick sampling surveys to better understand changes in the range of this species and its habitat and environmental preferences;
- Explore the potential for captive breeding and reintroduction of *I. nubecula* to its former sites and other suitable sites nearby;
- Develop and test environmental DNA technology to permit detection of *I. nubecula* eDNA from water samples;
- Perform a summer (low flow) habitat and nymph survey to identify specific habitat preferences, guide river restoration, and enable the communication of appropriate river management.

8. Acknowledgements

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10. Appendix 1. *Isogenus nubecula* and sampling site photographs.



Figure 4. *Isogenus nubecula* nymph from the River Dee in spring 2018.



Figure 5. River Dee at Froncysyllte (SJ 26760 42029) kick sampled 31st March 2018.



Figure 6. River Dee at Newbridge (SJ 28778 41666) kick sampled 15th March 2018.



Figure 7. River Dee at Coed Leodd (SJ 31349 39977) kick sampled 31st March 2018.



Figure 8. River Ceiriog u/s River Dee (SJ 31593 39155) kick sampled 11th March 2018.



Figure 9. River Dee at Erbistock (SJ 35423 41242) kick sampled 14th March 2018.



Figure 10. River Dee at Overton Bridge (SJ 35453 42744) kick sampled 14th March 2018.



Figure 11. River Dee at Bangor-on-Dee (SJ 38773 45480) kick sampled 9th March 2018.



Figure 12. River Dee at Pickhill Meadows (SJ 39725 46309) kick sampled 9th March 2018.



Figure 13. River Dee d/s River Clywedog (SJ 41320 47321) could not kick sample.



Figure 14. River Dee at Wern (SJ 41331 47665) kick sampled 1st April 2018.



Figure 15. River Alyn u/s River Dee (SJ 38236 56320) kick sampled 11th March 2018.



Figure 16. River Dee d/s River Alyn (SJ 40049 55715) could not kick sample.

11. Appendix 2. 2018 Survey Notes.

Site name Grid reference Samplers	Date visited	Genetics water samples	Site photo	Time sampling	Field notes
River Dee at Bangor on Dee SJ 38773 45480 John Davy-Bowker Mike Howe Michael Sweet Quentin Mauvisseau Chris Troth	9 Mar 2018	✓	✓	2 hours	<ul style="list-style-type: none"> • Approached the river from the south bank. • All large Perlodidae nymphs were found away from the river margins in fast water on coarse gravel. • Large Perlodidae were kept alive for adult rearing. • Small Perlodidae nymphs were preserved in Ethanol for Mike Hammett to examine. • <i>Rhithrogena germanica</i> mayfly nymphs present at the site. • One of the candidate <i>Isogenus nymphs</i> caught was observed eating a large <i>Rhithrogena germanica</i> mayfly nymph in the sorting tray. • Nineteen <i>Perlodes mortoni</i> and sixteen candidate <i>Isogenus nubecula</i> found.
River Dee at Pickhill Meadows SJ 39725 46309 John Davy-Bowker Mike Howe Michael Sweet Quentin Mauvisseau Chris Troth	9 Mar 2018	✓	✓	1 hour	<ul style="list-style-type: none"> • Approached the river from the south bank. • All large Perlodidae nymphs were found away from the river margins in fast water on coarse gravel. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • <i>Rhithrogena germanica</i> mayfly nymphs present at the site. • One of the candidate <i>Isogenus nymphs</i> caught was observed eating a large <i>Rhithrogena germanica</i> mayfly nymph in the sorting tray.
River Dee at Froncysyllte SJ 27033 42010 John Davy-Bowker	10 Mar 2018	✘	✓	-	<ul style="list-style-type: none"> • Site visited but could not sample due to excessively high river level (Manley Hall gauge = 1.03m)
River Ceiriog u/s River Dee SJ 31593 39155 John Davy-Bowker	11 Mar 2018	✓	✓	1½ hours	<ul style="list-style-type: none"> • Approached the river from the north bank. • River level had been high but fallen a little at the time of sampling (Manley Hall gauge = 1.00m). Water was deep and current was fast. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • <i>Rhithrogena germanica</i> mayfly nymphs present at the site.

<p>River Alyn u/s River Dee SJ 38236 56320 John Davy-Bowker</p>	<p>11 Mar 2018</p>	<p>✓</p>	<p>✓</p>	<p>¾ hour</p>	<ul style="list-style-type: none"> • Firstly, visited the former site at SJ 395 561 but the River Alyn at this location was very deep and the habitat was not judged to be suitable for <i>Isogenus nubecula</i>. • Then found a better site upstream at SJ 38236 56320, a riffle just downstream of a stone bridge, and sampled here instead. • Approached the river from the south bank. • River depth here on the River Alyn was fine for kick sampling (Manley Hall gauge = 0.97m). • Habitat was good with gravel and fast flowing water. • No Perlodidae stonefly species found. • Site was dominated by <i>Gammarus</i> shrimps and Baetidae mayflies, plus some <i>Rhyacophila</i> Caddis Flies. • Quite a few <i>Tubifex</i> worms were found stuck in the sampling net suggesting a water quality problem. • Macroinvertebrate fauna seemed more likely limited by water quality than by habitat.
<p>River Dee at Erbistock SJ 35423 41242 John Davy-Bowker Mike Howe</p>	<p>12 Mar 2018</p>	<p>✗</p>	<p>✗</p>	<p>-</p>	<ul style="list-style-type: none"> • Approached the river from the North bank. • Site visited but abandoned sampling due to high river level (Manley Hall gauge = 1.00m). • Water was very turbid following overnight rain.
<p>River Dee at Pickhill Meadows SJ 39725 46309 John Davy-Bowker Mike Howe</p>	<p>12 Mar 2018</p>	<p>✗</p>	<p>✗</p>	<p>1 hour</p>	<ul style="list-style-type: none"> • Return visit to Pickhill Meadow to collect live <i>Isogenus nubecula</i> nymphs for captive rearing. • Approached the river from the south bank. • River was deep (Manley Hall gauge = 1.06m to 1.09m). • Kick sampled tight to the south bank margins in deep water using a dry suit with Mike Howe's help. • Large Perlodidae nymphs were found close to the river margins in fast deep water on coarse gravel. • Seven candidate <i>Isogenus nubecula</i> caught. • Large Perlodidae were kept alive for adult rearing.

River Dee at Erbistock SJ 35423 41242 John Davy-Bowker	14 Mar 2018	✓	✓	1½ hours	<ul style="list-style-type: none"> • Approached the river from the north bank. • River was deep (Manley Hall gauge = 0.91m) but the river looked more accessible than when it was visited on the 12th of March. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • One candidate <i>Isogenus nubecula</i> nymph was found. • <i>Rhithrogena germanica</i> mayfly nymphs present at the site.
River Dee at Overton Bridge SJ 35453 42744 John Davy-Bowker	14 Mar 2018	✓	✓	1½ hours	<ul style="list-style-type: none"> • Approached the river from the north bank. • River was deep (Manley Hall gauge = 0.89m). • Moved the site from its original location to a new site closer towards the bridge and sampled at SJ 35453 42744, a small area close to the bank near to the bridge. Access was better here during high flow conditions. • River access was still difficult. Could only sample in sub-optimal habitat comprising coarse blocks rather than gravel. • One possible <i>Isogenus nubecula</i> found but it was not possible to confirm this in the field (could be <i>Perlodes mortoni</i>). This was preserved in Ethanol for subsequent identification in the laboratory. • Only one <i>Rhithrogena germanica</i> mayfly nymph was found here.
River Dee at Newbridge SJ 28778 41666 John Davy-Bowker	15 Mar 2019	✓	✓	1½ hours	<ul style="list-style-type: none"> • Approached the river from the north bank. • Has rained overnight, but not a lot. • River was deep (Manley Hall gauge = 0.90m). River rising now. • A good gravel bed present that was easy to sample. • Large Perlodidae were found mainly away from the margin on coarse gravel in fast flow. • Lots of <i>Perlodes mortoni</i> stoneflies and <i>Rhithrogena germanica</i> mayfly nymphs were found. No candidate <i>Isogenus nubecula</i> were seen. Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory.

<p>River Dee d/s River Alyn SJ 40049 55715 John Davy-Bowker</p>	<p>15 Mar 2019</p>	<p>✓</p>	<p>✓</p>	<p>-</p>	<ul style="list-style-type: none"> • Site was extremely deep (Manley Hall gauge = 0.94m) and substratum appeared to be entirely composed of sand and/or silt. • It was not possible to kick sample even with a dry suit. • Explored area upstream on foot and no gravel beds found. • Looking at aerial imagery on Google Maps suggested there were no gravel beds for several miles upstream or downstream. All of the river around here seemed to have deep slow flowing water with no gravel beds.
<p>River Dee at Coed Leodd SJ 31349 39977 John Davy-Bowker</p>	<p>16 Mar 2018</p>	<p>✘</p>	<p>✘</p>	<p>-</p>	<ul style="list-style-type: none"> • Rained overnight and river rising. Manley Hall gauge = 0.89m at 09:00 and 1.03m at 13:45. • Original site at SJ 319 394 was very difficult to access and no gravel bed could be seen to kick sample. • Found a better site quite nearby. Access was complicated: drive in from Ruabon, Rhos-y-madoc and Pentre, park at SJ 31629 39871, just before a farm yard. Walk to the river Dee and then walk upstream to the site at SJ 31349 39977 (accessing the river from the north bank). • The river was still too high to sample on this occasion (Manley Hall gauge = 1.03m).
<p>River Dee at Froncysyllte SJ 26850 42062 John Davy-Bowker</p>	<p>16 Mar 2018</p>	<p>✘</p>	<p>✘</p>	<p>-</p>	<ul style="list-style-type: none"> • An alternative Froncysyllte site was found at SJ 26850 42062 just downstream of an old stone bridge with conditions better than the Froncysyllte site visited on the 10th of March. • Park nearby at SJ 26789 42078 then a short walk in. • Approached the river from the north bank. • River was also too deep to sample at this new site on the 16th of March (Manley Hall gauge = 1.03m). Site not sampled.

River Dee d/s River Clywedog SJ 41320 47321 John Davy-Bowker	16 Mar 2018	✘	✘	-	<ul style="list-style-type: none"> • Explored southern approach to this site. Road access poor but accessible with a long walk in. • Best option is to drive in from Worthenbury, than park on a track at SJ41818 46397. Walk in taking equipment to sample this site and the Wern site • Manley Hall gauge = 1.18m. • River too deep to sample.
River Dee at Wern SJ 41331 47665 John Davy-Bowker	16 Mar 2018	✘	✘	-	<ul style="list-style-type: none"> • Explored southern approach to this site. Road access poor but accessible with a long walk in. • Best option is to drive in from Worthenbury, than park on a track at SJ41818 46397. Walk in taking equipment to sample this site and the Dee d/s Clywedog site. • Manley Hall gauge = 1.18m. • River too deep to sample.
River Dee at Froncysyllte SJ 26760 42029 John Davy-Bowker	31 Mar 2018	✓	✓	1 hour	<ul style="list-style-type: none"> • Returned for a second fieldwork visit to North Wales to attempt to fill in missing sites that could not be sampled previously due to high river levels. • River Dee has been low for several days but has rained overnight and river has risen again this morning. Manley Hall gauge = 0.83m. • Approached the river from the north bank. • Searched for a better site and sampled at SJ 26760 42029, just upstream of the bridge and downstream of a wooden house. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • No candidate <i>Isogenus nubecula</i> seen. • <i>Rhithrogena germanica</i> present at the site.
River Dee at Coed Leodd SJ 31349 39977 John Davy-Bowker	31 Mar 2018	✓	✓	1 hour	<ul style="list-style-type: none"> • Approached the river from the north bank. • River has risen again. Manley Hall gauge = 0.97m. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • No candidate <i>Isogenus nubecula</i> seen. • <i>Rhithrogena germanica</i> present at the site.

River Dee at Wern SJ 41331 47665 John Davy-Bowker	1 Apr 2018	✓	✓	1 hour	<ul style="list-style-type: none"> • Approached the river from the south bank (see important notes from the 16 Mar 2018 regarding accessing this site at the same time as the River Dee d/s River Clywedog site). • This site can only be accessed from the south. • Dry weather and river has dropped again a little. Manley Hall gauge = 0.88m. • Walked round a large bend in the river and sampled an accessible sand bar on the inside of the bend. • Few riffles and lots of sand. Coarse substrate hard to find. • Large Perlodidae were preserved in Ethanol for subsequent identification in the laboratory. • One candidate <i>Isogenus nubecula</i> seen (subsequent Lab ID of large Perlodidae confirmed three <i>Isogenus nubecula</i>). • <i>Rhithrogena germanica</i> present at the site.
River Dee d/s River Clywedog SJ 41320 47321 John Davy-Bowker	1 Apr 2018	✓	✓	-	<ul style="list-style-type: none"> • Approached the river from the south bank (see important notes from the 16 Mar 2018 regarding accessing this site). • This site can only be accessed from the south. • Dry weather. Manley Hall gauge = 0.86m. • No riffles. River quite fast. River deep. Streambed inaccessible. Where I could push a pole into it from the river bank it seemed to be composed of sand rather than gravel. • Too deep to take an invertebrate sample.
River Dee at Pickhill Meadows SJ 39725 46309 John Davy-Bowker	1 Apr 2018	✗	✗	1 hour	<ul style="list-style-type: none"> • Approached the river from the south bank. • Return visit to Pickhill Meadows for last collection of live <i>Isogenus nubecula</i> nymphs for adult rearing. • Collected good numbers of Perlodidae nymphs: sixteen <i>Perlodes mortoni</i> and thirty two candidate <i>Isogenus nubecula</i>. • Bad weather on the way (snow/rain). End of second fieldwork trip to North Wales.

12. Data Archive Appendix

The data archive contains:

[A] The final report in Microsoft Word and Adobe PDF formats.

[B] Species records, which are held on the NRW Recorder 6 database.

Metadata for this project is publicly accessible through Natural Resources Wales' Library Catalogue <http://libcat.naturalresources.wales> or <http://catllyfr.cyfoethnaturiol.cymru> by searching 'Dataset Titles'. The metadata is held as record no 122445.



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