

# The National Crayfish Plague Surveillance Programme, Ireland – 2020-2021



*White-clawed crayfish. Photo: B.Nelson*



*Foras na Mara*  
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An tSeirbhís Páirceanna Náisiúnta  
agus Fiadhúlra  
National Parks and Wildlife Service

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Please refer to the documents "Report National Crayfish Plague Surveillance program 2018\_2019" and "NCPSP proposal 2020\_2022" for additional/background information.

## Abbreviations

NCPSP	National Crayfish Plague Surveillance Programme
CFP	Crayfish plague/ <i>Aphanomyces astaci</i>
WCC	White-clawed crayfish ( <i>Austropotamobius pallipes</i> )
qPCR	Taqman real-time PCR
eDNA	environmental DNA
NICS	Non-Indigenous Crayfish Species
CMC	Chinese Mitten Crab

## 1 Executive Summary

Following on from the successful completion of the first National Crayfish Plague Surveillance Programme (NCPSP 2018-2019), a second programme was established in 2020 (NCPSP 2020-2021). A similar project design and work programme were applied, but this programme also incorporated some additional studies of disease persistence and prevalence using a time series study of two infected catchments at two time points over the two years. Ecological field survey assessments were also planned for eDNA sampling sites to allow for a comparison of more traditional field ecology surveys with the newer molecular detection techniques.

The collected data suggest a seasonal effect on the detection of both disease and crayfish as the number of positive detections increased during November. NPWS completed ecological field survey assessments in approximately the same area as eDNA sampling sites, and the results are comparable for all sites where we have information for both. In fact, the eDNA detection method arguably outperformed the more traditional field survey method in multiple locations. At the conclusion of the second programme, no invasive crayfish species were detected in any crayfish plague-positive catchments tested using the novel multiplex assay.

The results of the NCPSP 2020-2021 further demonstrate a clear and continued spread of crayfish plague both within and between the river catchments of Ireland surveyed. The number of infected sites increased by 59% between the first and second programme, with three additional catchments now classed as CFP-positive since the conclusion of NCPSP 2018-2019. However, the overall WCC population is still healthy in many catchments, with only two of the twenty-seven surveyed failing to record a single positive site. A comparison of the WCC population density during both programmes seems to indicate the crayfish are maintaining a steady presence within these river catchments despite the continued threat of crayfish plague.

## 2 Introduction

*Aphanomyces astaci*, the causative agent of crayfish plague (CFP), was first confirmed present in Ireland by molecular detection techniques in 2015 following mass mortality of White-clawed crayfish (WCC) (*Austropotamobius pallipes*) in the River Bruskey, Co. Cavan. A number of separate mortality events followed over the next three years, and in 2018 the first National Crayfish Plague Surveillance Programme (NCPSP 2018-2019) commenced as a collaborative effort between the Marine Institute and National Parks and Wildlife Service. This pioneering programme used environmental DNA (eDNA) taken directly from waterways to screen for the presence of CFP and WCC across the river catchments of Ireland with suitable crayfish habitats. This programme was extremely effective, with both pathogen and native crayfish successfully detected at numerous sites throughout Ireland, with detection of plague possible in the absence of any crayfish mortality. A novel multiplex assay to detect 8 Non-Indigenous Crayfish Species (NICS) was also developed during this programme. No positive NICS detections were recorded in any of the crayfish plague-infected catchments tested.

The first surveillance program (NCPSP 2018-2019) set out to establish the prevalence of CFP across the catchments of Ireland believed to still have WCC populations, making use of the latest molecular technology to detect pathogen DNA signatures from the aquatic environment using eDNA sampling and real time PCR methods. In addition, the simultaneous detection of WCC and eight NICS was also possible. A detailed report on the methods and outcomes of the NCPSP 2018-2019 can be downloaded from <https://www.fishhealth.ie/fhu/news-media/news/update-crayfish-plague-ireland-0>. This report also provides background information on Irish WCC populations, CFP and its impact and the establishment of the project. The first programme provided excellent evidence for the use of environmental DNA (eDNA) as a surveillance tool for both CFP and WCC, with new CFP-positive catchments identified from eDNA alone with no associated evidence of crayfish mortalities. eDNA detection of WCC was also possible.

This programme, the second NCPSP 2020-2021, was a continuation and extension of the first programme using the same sampling and testing regime, with some adjustments to site numbers related to catchment size and disease status. This programme also incorporated additional studies of disease persistence and prevalence using a time series study of two infected catchments at two time points over the two years and also a comparison of field survey results with molecular detection results.

The key areas of collaboration covered by the programme were:

1. the investigation of crayfish mortalities in Ireland, using appropriate testing methods to determine if crayfish plague is the causative agent in these mortality events
2. refinement of eDNA sampling methods and real-time PCR methods to detect presence or absence of Crayfish Plague and selected non-indigenous crayfish species in Irish freshwaters
3. undertake surveillance using a combination of eDNA methods and field survey of the White-clawed Crayfish throughout its known habitats in Ireland
4. investigate the catchments with Crayfish Plague outbreaks to determine the extent and persistence of the outbreak
5. disseminate information on the Crayfish Plague in Ireland including in scientific peer-review journals
6. investigation of the vectors of introduction and
7. conservation strategies for the White-clawed Crayfish in Irish freshwaters

### 3 Work Programme Summary

#### 3.1 Field sampling:

Details of the catchments and sites surveyed, in which year(s) and the number of water samples taken, are recorded in Table 1.

Table 1: Summary of eDNA samples collected during the NCPSP 2020-2021.

	Catchment Details	No. of Sites	No. of water samples	Year 1 2020	Year 2 2021
	<b>Time Series</b>				
1	Shannon Estuary South	12 (+ 1 extra)	36 (+3)	✓	✓
2	Barrow	12	36	✓	✓
	<b>CFP Positive</b>				
3	Erne	15	45	✓	
4	Nore	11	33	✓	
5	Suir	10	30	✓	
6	Corrib	10	30	✓	
7	Shannon 26A	6	18		✓
8	Shannon 26B	6	18		✓
9	Shannon 26D	8	24		✓
10	Shannon 26G	7	21	✓	
11	Shannon 25C	7	21	✓	
12	Ulster Blackwater	6	18	✓	
	<b>CFP negative</b>		120		
13	Boyne	4	12	✓	
14	Munster Blackwater	4	12	✓	
15	Moy	4 (+3 extra)	12 (+9)	✓	✓
16	Sligo	4 (+3 extra)	12 (+9)	✓	✓
17	Shannon 25A	6	18	✓	
18	Shannon 25B	4	12		✓
19	Shannon 25D	4	12		✓
20	Shannon 26C	4	12		✓
21	Shannon 26E	4	12		✓
22	Ballintra	4	12	✓	
23	Liffey	5	15		✓
24	Grand Canal	4	12		✓
25	Clarín/Kilcolgan/Kinvarra	6	18		✓
	Totals	168	738		

#### 3.2 Laboratory Processes:

A detailed description of the materials, methods and associated references is available in the NCPSP 2018-2019 final report <https://www.fishhealth.ie/fhu/news-media/news/update-crayfish-plague-ireland-0>.

- All 738 water samples were processed using the modified Qiagen Power Water kit eDNA extraction protocol.
- All eDNA samples were tested to detect the presence of CFP and WCC in a single combined Taqman real time PCR (qPCR) reaction.
- All sites from CFP positive catchments were tested to detect the presence of NICS (8 crayfish species) using three qPCR multiplex reactions, which detect 2 or 3 species simultaneously.
- Ecological field survey data collated and compared with NCPSP WCC eDNA results.
- Genotyping of all mortality samples complete.
- Limits of detection, sensitivity and specificity of CFP and WCC qPCR method determined. (see section 4.7 for further details)

## 4 Results and Outcomes

### 4.1 CFP detections

Altogether, over the course of the 2020-2021 programme, eDNA extracted from water samples at 168 sites sampled once and 30 sites sampled twice was tested to detect the presence of CFP and WCC in triplicate.

Twenty-nine individual sites in nine different catchments tested positive for the presence of CFP. Twenty-three of these sites were confirmed CFP-positive for the first time during this programme. Three of the nine catchments, Sligo, Moy and Shannon 26C, tested positive for the presence of CFP for the first time during this programme (Table 2).

During the 2018-2019 surveillance programme, a CFP-positive eDNA sample was recorded at one site in the Shannon 26A catchment at Ballyfarnon on the Feorish River. All sites in this catchment are now CFP-negative.

All NCPSP 2020-2021 positive eDNA CFP detections are detailed in Table 2, and all CFP detections to date, new and historical, related to eDNA or mortality, are mapped in Figure 1.

There were also four WCC mortality events between 2020 and 2021. A single crayfish mortality event tested positive for the presence of CFP in 2020, and three CFP-related mortality events occurred in 2021.

In Shannon Estuary South:

- 2020: River Camogue at Manister Abbey, Mague, Co. Limerick, 30/30 tested positive (S/4/20)
- 2021: River Loobagh, Mague, Co. Limerick, 30/30 tested positive (S/9/21)
- 2021: River Mague, Ballinahinch Bridge, Co. Limerick, 7/7 tested positive (S/16/21)

In Shannon 25A:

- 2021: River Clodiagh, Clonaslee, Co. Laois, 4/4 tested positive (S/31/21)

Table 2: Sites and catchments confirmed positive for the presence of CFP from NCPSP 2020-2021 eDNA data.

Site	Catchment	Site Name	Sample Reference	River/Lake	CFP Mean Ct	WCC Mean Ct	Additional details
1	Shannon ES	Askeaton Main Street	E_3_20_S5	Deel	36.06	40.38	
	Shannon ES	Askeaton Main Street	E_18_20_S5	Deel	37.27	negative	
2	Shannon ES	Athlaca (Howardstown)	E_3_20_S8	Morning Star	26.73	26.75	
	Shannon ES	Athlaca (Howardstown)	E_18_20_S8	Morning Star	32.63	36.26	
3	Shannon ES	Ballinahinch Bridge	E_3_21	Morning Star	30.18	31.65	Mortality site S/16/21
4	Shannon ES	River Camogue, Manister	E_3_20_S9	Camogue	27.06	27.48	
	Shannon ES	River Camogue, Manister	E_18_20_S9	Camogue	29.71	35.58	
5	Shannon ES	Croom	E_3_20_S10	Maigne	33.8	34.98	
	Shannon ES	Croom	E_18_20_S10	Maigne	31.38	35.56	
	Shannon ES	Croom	E_18_21_S10	Maigne	38.39	37.22	
6	Shannon ES	Castleroberts Bridge	E_3_20_S11	Maigne	35.08	37.78	Also detected in 2019
	Shannon ES	Castleroberts Bridge	E_18_20_S11	Maigne	31.39	36.94	Also detected in 2019
7	Shannon ES	River Loobagh	E_18_20_S6	Loobagh	37.38	33.11	
	Shannon ES	River Loobagh	E_1_21_S6	Loobagh	38.94	34.47	
	Shannon ES	River Loobagh	E_18_21_S6	Loobagh	34.3	34.29	
8	Shannon ES	Bruree	E_18_20_S7	Maigne	35.17	35.75	
9	Shannon ES	Adare	E_18_20_S12	Maigne	32.83	37.38	Also detected in 2019
	Shannon ES	Adare	E_18_21_S12	Maigne	37	37.19	Also detected in 2019
10	Barrow	Two Mile Bridge	E_4_20_S1	Barrow	35.98	negative	
	Barrow	Two Mile Bridge	E_19_20_S1	Barrow	35.93	negative	
11	Barrow	Owenass	E_4_20_S2	Owenass	30.89	33.78	
12	Barrow	Millgrove	E_19_20_S3	Figile	36.98	37.6	
13	Barrow	Monasterevin	E_19_20_S5	Barrow	36.23	38.11	Also detected in 2018
14	Barrow	Leighlinbridge	E_19_20_S9	Barrow	36.2	negative	
15	Nore	Newbridge Cloncough	E_5_20_S1	Nore	37.94	35.7	Also detected in 2019
16	Nore	Three Castles bridge	E_5_20_S4	Nore	38.57	36.29	
17	Nore	Jenkinstown Park	E_5_20_S5	Dimin	37.19	36.13	
18	Suir	Ballygriffin	E_6_20_S3	Multeen	36.19	negative	Also detected in 2018
19	Corrib	D/S Corrofin	E_7_20_S7	Clare	36.7	35.39	Also detected in 2018 & 2019
20	Moy	Cloonacannana	E_10_20_S1	Moy	35.82	34.78	
	Moy	Cloonacannana	E_17_21_2	Moy	35.74	35.53	
21	Moy	Upstream POS 2020	E_17_21_1	Moy	34.8	33.3	
22	Moy	D/S positive 2020	E_17_21_3	Moy	35.62	35.25	
23	Sligo	Gurteen	E_13_20_S1	Owenmore	34.8	33.35	
	Sligo	Gurteen	E_16_21_1	Owenmore	29.97	32.63	
24	Sligo	Boyle Road, Gurteen	E_16_21_2	Owenmore	30.95	35.88	
25	Sligo	Gurteen 2	E_16_21_3	Owenmore	31.73	32.03	
26	Shannon 26B	Bridge W of Ballandareen	E_12_21_2	Lung River	35.27	36.64	
27	Shannon 26B	Boyle Footbridge	E_12_21_5	Boyle River	33.3	36.03	
28	Shannon 26C	Drumsna	E_15_21_1	Shannon	37.03	negative	
29	Shannon 26C	Rinn Marina	E_15_21_2	Rinn River	38.01	negative	

Note: E\_3 and E\_4 refer to July sampling. E\_18 and E\_19 refers to November sampling. The second number in the sample reference refers to the year the sample was taken e.g. E\_3\_20\_S5 was taken in 2020, E\_15\_21\_2 was taken in 2021. Shannon ES = Shannon Estuary South.

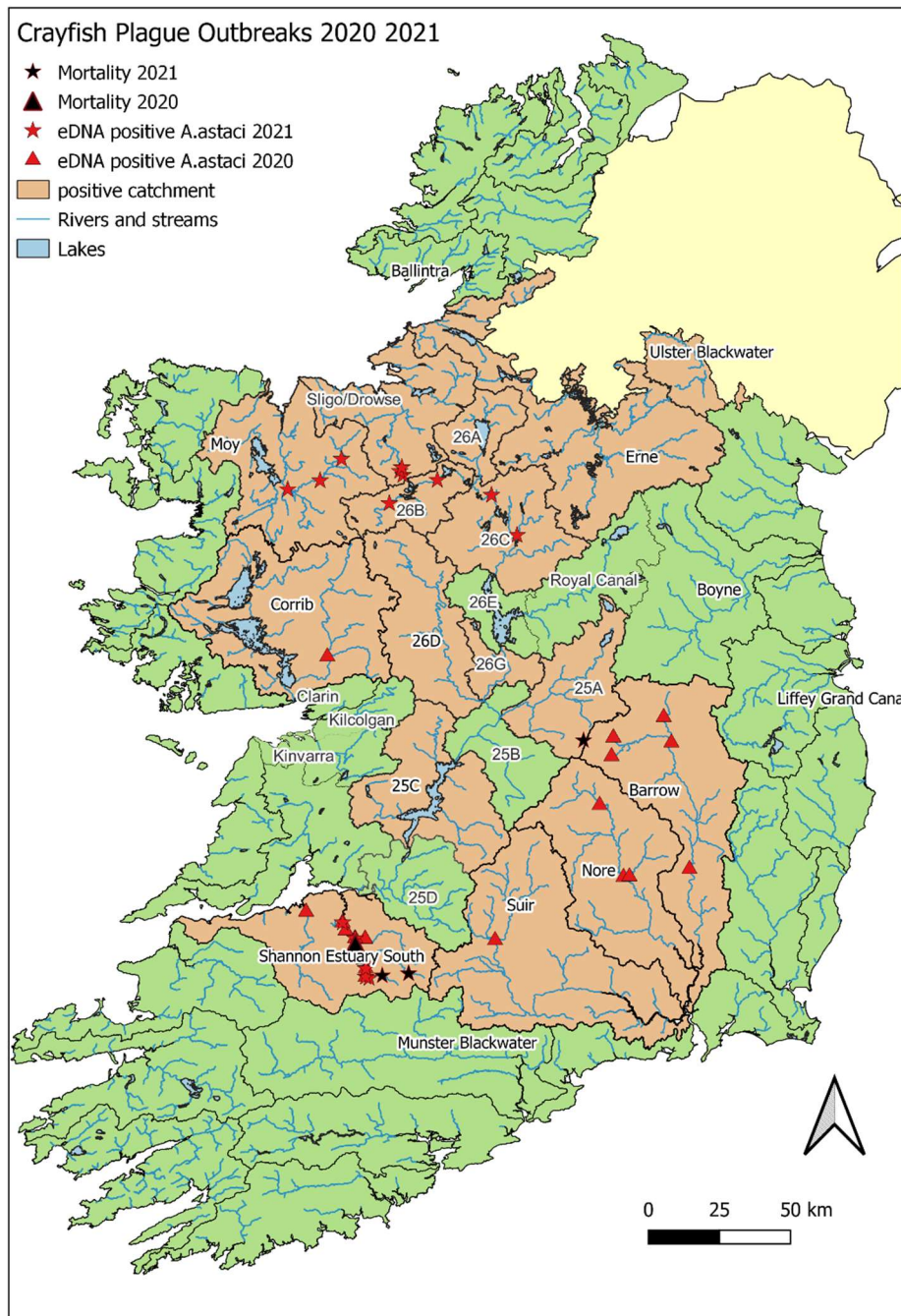


Figure 1: Map of 2020 and 2021 sampled catchments with positive CFP results indicated for both eDNA (red) and mortality samples (black) for each year. Catchments confirmed as CFP positive by mortality or eDNA data are highlighted in orange.

#### 4.2 WCC detections

For most catchments (22/27; 81.5%), the presence of WCC DNA was detected at multiple sites with the exception of the Barrow, Suir, Grand Canal, Shannon 25D and 26C catchments. In 2020, positive WCC detections were recorded at only one site in the July sampling in the Barrow and two sites in the Suir catchment. Positive WCC detection did increase to three sites in the Barrow catchment when sampled again in November, however, we did not detect WCC in the Barrow catchment in either July or November in 2021. In 2021, positive WCC detections were recorded at only one site in the Grand Canal catchment. All four sites surveyed were negative in both the Shannon 25D and 26C catchments surveyed in 2021. Both of these catchments are also crayfish-plague negative.

The map in Figure 2 illustrates the density of WCC detections in each catchment sampled in 2020 and 2021, with a weighted percentage of positive sites used to produce the colour gradient.

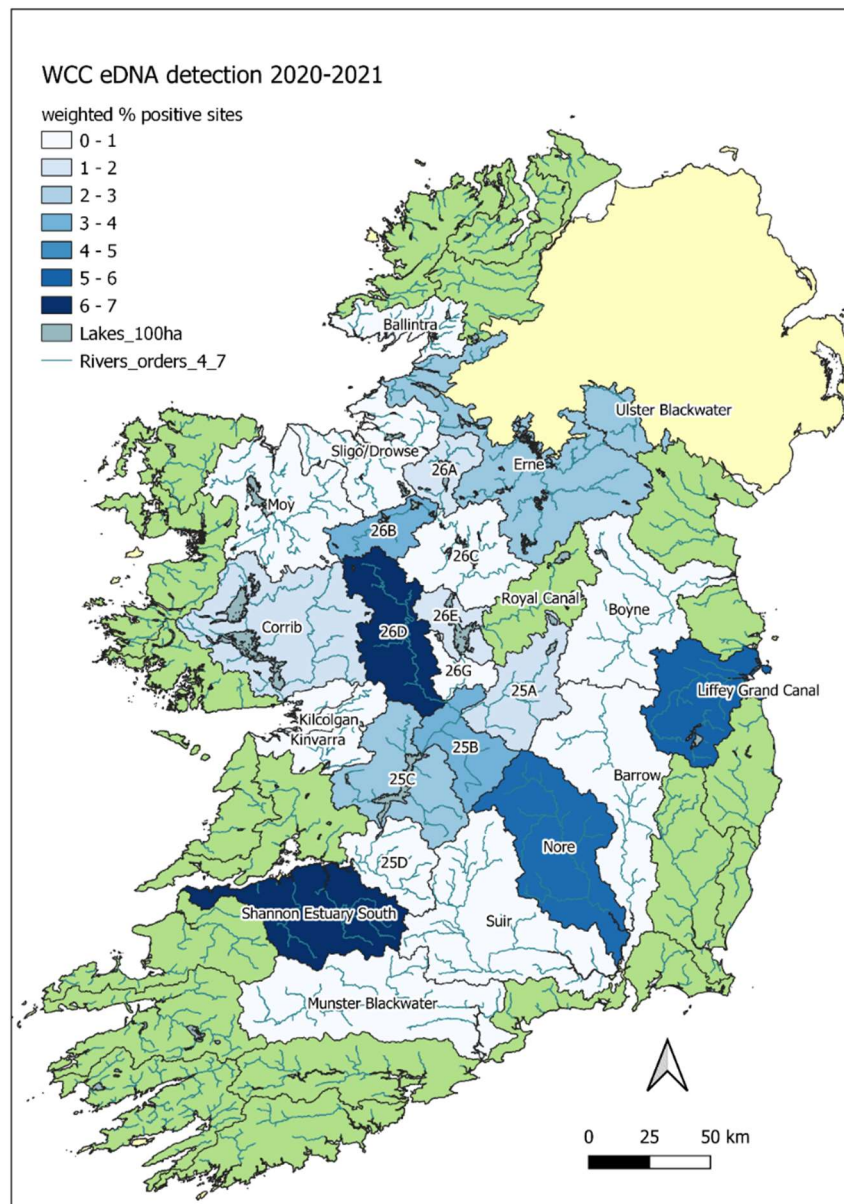


Figure 2: Map of 2020 and 2021 sampled catchments with positive WCC results indicated. Colour gradient is calculated using the % of positive sites in that catchments with darker blue colour, indicating a higher percentage of positive sites. A weighted calculation was used to account for the varying number of sites in each catchment.

### 4.3 NICS non-detections

All eDNA samples from each CFP-positive catchment were tested to detect the presence of 8 different crayfish species using a series of qPCR multiplex assays. A multiplex assay can distinguish between and detect the presence of three distinct species in a single reaction. No evidence of the presence of NICS was detected in any samples tested to date.

### 4.4 Ecological field surveys

Four regions were selected by NPWS for field ecology studies to determine the density of WCC using the traditional field survey methods of hand searches and net sweeps. Three of the four areas selected were also eDNA sampled in 2020, however, the region of the North Midland lakes was eDNA sampled in 2021.

A comparison of the 2 methods is presented in Table 3; in some regions, the results do not refer to exactly the same sites, so we have chosen the closest eDNA surveillance site for some comparisons.

Overall there is very strong agreement between the two methods, with eDNA detection possible for every field ecology detection with the exception of one site in Lough O' Flynn (Table 3). At four of the field ecology sites, a negative result was recorded, but eDNA detection was possible, highlighting the extra sensitivity of the molecular detection method. As detection of historical or "old" WCC eDNA signatures is also possible, this is an additional advantage for molecular detection methods when compared to field ecology.

Table 3: NPWS WCC field ecology survey data and comparison with closest eDNA sampling sites where available.

Region	Field Ecology Site	WCC found at site?	WCC eDNA found at site?	Comparable WCC eDNA site surveillance site
<b>South Donegal Lakes</b>				
Lough Nageage SAC	Lough Nageage Site 03	Negative	Negative	Lough Nageage
Lough Nageage SAC	Lough Naveane Site 01	Negative	Negative	Lough Naveane
Lough Nageage SAC	Lough Veenagreane Site 02	Positive	Positive	Lough Veenagreane
<b>Ballintra</b>				
Ballintra River	Aghadullagh Bridge Site 01	Negative	Positive	Aghadullagh
Ballintra River	Rath Lough Bridge Site 01	Negative	Positive	Lough Rath
<b>Erne</b>				
Erne	ANN01	Positive	Positive	Br SE of Fort William, Cootehill
Erne	DRO01	Negative	Negative	Ballynascarva Br
Erne	CAV01	Positive	Positive	Lisdam, Cavan Town
<b>Ulster Blackwater</b>				
Blackwater	MOU01	Positive	Positive	Bridge N/E of Golden
Blackwater	MOU02	Positive	Positive	Emyvale
Blackwater	BLA01	Positive	Positive	Scotstown
Blackwater	BLA02	Positive	Positive	Newmills Bridge
<b>Midlands Lakes</b>				
Lough Nanannagh	NAN_LNAN_01	Positive	Positive	Nannannagh Lough
Lough Nanannagh	NAN_STRE_02	Negative	Positive	Lough Nanannagh stream
Lough O'Flynn	OFL_LOFL_02	Positive	Positive	Lough O'Flynn 2B
Lough O'Flynn	OFL_RSUC_01	Negative	Positive	Lough O'Flynn 2C
Lough O'Flynn	OFL_RSUC_02	Positive	Negative	Lough O'Flynn 2D

#### 4.5 Time Series

A time series investigation commenced in 2020, with 12 sites in both the Barrow and Shannon Estuary South catchments sampled twice in both July and November in 2020 and 2021. This study aimed to assess disease incidence in these areas, as multiple CFP-related crayfish mortalities had been recorded in these catchments, and also determine disease persistence over time.

The results are somewhat inconsistent and raise some interesting questions regarding negative results.

For CFP detection, the results from the 2020 experiment indicate an apparent increase in the number of sites recorded as CFP-positive during the November sampling time point when compared to July for both catchments. In the Shannon Estuary South catchment, there were five sites positive in July, which increased to eight sites positive in November. In the Barrow, there were two sites positive in July, which increased to four sites in November. In the Barrow, one site of the 12 tested was positive in July and then found to be negative in November. However, in 2021, while the results from Shannon Estuary South followed the same pattern as the year before, CFP was not detected at any site in the Barrow in either July or November.

For the WCC detections, the pattern of results is not as obvious, with sites in both catchments changing from positive and negative in July to vice versa in November. In 2020, one site in Shannon Estuary South and three sites in the Barrow changed from positive detection in July to non-detected in November. There was one site in each catchment which was positive in July and negative in November. Overall in 2020, the total number of positive WCC detections increased in the Barrow from one site to three sites but remained unchanged in Shannon Estuary South. In 2021 the Shannon Estuary South results followed the same pattern as found in 2020, with the number of WCC-positive sites increasing in November, however, WCC was not detected in the Barrow. See Table 4 for details. The 2021 results from the Barrow are unexpected and warrant further detailed investigation.

Table 4 : Comparison of results from July and November to detect both WCC and CFP in the Barrow and Shannon Estuary South catchments in 2020 and 2021. SES=Shannon Estuary South

	2020		2021	
	SES	Barrow	SES	Barrow
<u>Crayfish Plague detections</u>				
<b>Total No. Positive Sites - July</b>	5	2	1	0
<b>Total No. Positive Sites - Nov</b>	8	4	4	0
<b>Positive (July) to negative (Nov) sites</b>	0	1	0	0
<b>Negative (July) to positive (Nov) sites</b>	3	3	3	0
<u>White-clawed crayfish detections</u>				
<b>Total No. Positive Sites - July</b>	9	1	2	0
<b>Total No. Positive Sites - Nov</b>	9	3	6	0
<b>Positive (July) to negative (Nov) sites</b>	1	1	0	0
<b>Negative (July) to positive (Nov) sites</b>	1	3	4	0

#### 4.6 CFP Genotypes

Following extensive optimisation of the microsatellite marker method in-house and addition of a commercially available clean-up kit to the protocol, genotype information was obtained for both historical and current CFP-positive samples. The qPCR genotyping method, as described by (Di Domenico et al., 2021) was also used to determine isolate genotypes.

All samples from the Erne catchment taken during the 2015 CFP outbreak and subsequent 2016 survey by GMIT (Mirimin et al., 2022) have now recorded a B genotype which originates from the Californian Signal crayfish. One set of samples (S/94/15), taken from the first mortality samples, shows evidence of both genotypes B and C being present in the same sample. These genotypes originate from the same species of crayfish and differ only in the original geographic location, B is associated with a Californian Signal crayfish subtype, and C is related to the original Canadian Signal crayfish subtype.

During the NCPSP 2018-2019, Shannon 26A and 26D were classed as CFP positive from eDNA samples only with unknown genotypes. From work undertaken in this programme, we can now designate Shannon 26A as a D genotype, although the subtype is still unknown.

There is now evidence for the presence of both A and D genotypes in the Shannon 26D, Sligo and Moy catchments. Interestingly, all three catchments are in close proximity to the Corrib catchment, which has previously recorded both A and D genotypes from separate eDNA samples taken at the same site in 2018 and 2019. The presence of CFP isolates with different genotypes is now evident in the Corrib, Sligo, Moy and Shannon 26D catchments.

There was a single mortality sample (S/4/20) recorded in 2020 on the River Camogue, close to the River Maigue in the Shannon Estuary South catchment, which also recorded a D genotype, the eDNA sample (E\_1\_20) from that mortality event was also confirmed as Genotype D. Mortality isolates S/9/21 and S/16/21, both also taken from the River Maigue, are also confirmed D genotypes although subtype information is not available. This is in line with the genotype expected to be present in the Shannon Estuary South catchment.

The CFP isolate from the mortality sample in Shannon 25A is confirmed as Genotype D. The mortality occurred very close to the border with the Barrow catchment, which is also Genotype D. We can now designate Shannon 26B as genotype A.

A map of Ireland with genotypes indicated for each catchment is presented in Figure 3. See Table 5 for details within catchments.

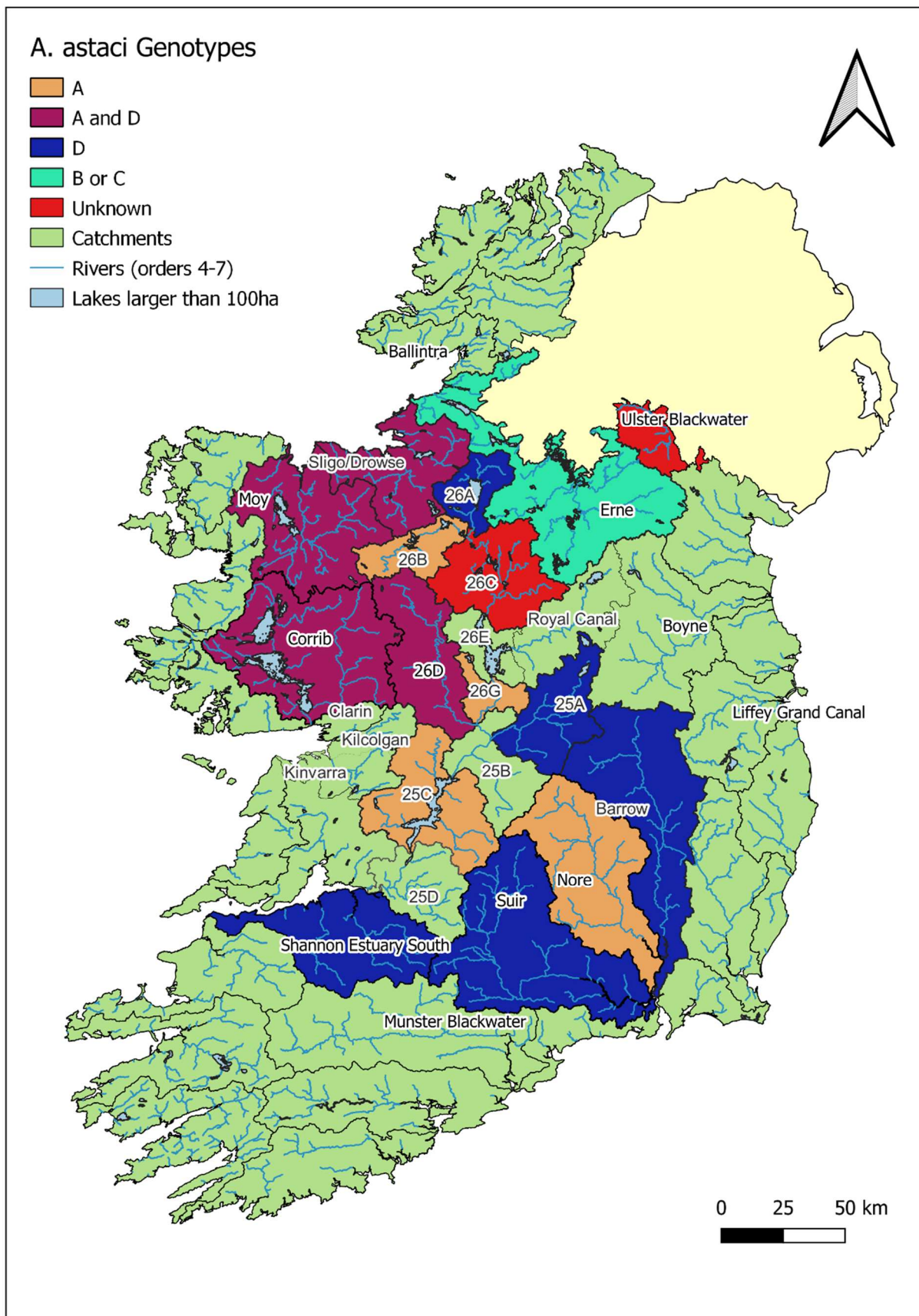


Figure 3: Catchment map of Ireland with crayfish plague isolate genotypes indicated.

Table 5: Genotype details, where available, for CFP-infected catchments.

Catchment	River	Year	FHU Sample ID	Genotype	Crayfish Origin
<b>Erne</b>	Bruskey	2015	S/94/15	B and C (evidence for both)	Californian Signal/Canadian Signal
		2015	S/88/15	B indicated (incomplete data)	Californian Signal
		2015	S/104/15	B indicated (incomplete data)	Californian Signal
		2016	S/63/16	B indicated (incomplete data)	Californian Signal
<b>Suir</b>	Suir	2017	S/21/17	D, subtype D1	American Red-swamp
<b>Shannon 25C</b>	Lorrha	2017	S/63/17	A	European Noble/Turkish
<b>Shannon ES</b>	Deel	2017	S/83/17	D, subtype D2	American Red-swamp
	Maigue	2019	S/5/19	D, subtype D1	American Red-swamp
	Maigue	2020	S/4/20 & E/1/20	D, subtype unknown	American Red-swamp
	Maigue	2021	S/9/21	D, subtype unknown	American Red-swamp
	Maigue	2021	S/16/21	D, subtype unknown	American Red-swamp
<b>Barrow</b>	Barrow	2017	S/74/17	D, subtype D2	American Red-swamp
	Slate	2019	S/8/19	D, subtype D1	American Red-swamp
<b>Corrib</b>	Clare	2018	E/C/18/S8	A	European Noble/Turkish
		2019	E/1/19_S8F2	D, subtype unknown	American Red-swamp
<b>Shannon 26G</b>	Al	2018	S/86/18	A	European Noble/Turkish
<b>Ulster Blackwater</b>	Ulster Blackwater	2018	S/64/18	Unknown	
<b>Shannon 26D</b>	Suck	2019	E/4/19_S3F3	A or D, subtype unknown	European Noble/Turkish/American Red-swamp
<b>Shannon 26A</b>	Feorish	2019	E/15/19_S3F2_F3	D, subtype unknown	American Red-swamp
<b>Nore</b>	Nore	2019	S/42/19	A	European Noble/Turkish
<b>Shannon 26B</b>	Shannon	2019	E/5/19_S6	A	European Noble/Turkish
<b>Shannon 25A</b>	Clodiagh	2021	S/31/21	D, subtype unknown	American Red-swamp
<b>Moy</b>		2021	E/17/21	A and D (evidence for both)	European Noble/Turkish/American Red-swamp
<b>Sligo</b>		2021	E/16/21	A and D (evidence for both)	European Noble/Turkish/American Red-swamp

Note: S sample IDs are mortality samples, and E sample IDs are eDNA samples.

#### 4.7 qPCR Method Validation

Validation experiments to determine the limits of detection (LOD), sensitivity and reproducibility of the qPCR methods to detect CFP and WCC were completed, both in singleplex and duplex reactions. A plasmid containing the sequence targeted by the qPCR assay was constructed for both the CFP and WCC tests. Plasmid can be quantified precisely and is useful to determine the exact DNA copy number present at the limit of detection. Plasmid does not always behave the same as real world samples, so we have also tested CFP-infected tissue and plasmid that has been spiked into real world samples i.e. processed through the eDNA extraction protocol for comparison. The same tissue samples were used for both the CFP and WCC validation experiments and also to test the duplex assay, which targets both CFP and WCC simultaneously.

The results indicate both assays perform extremely well and are reproducible even at very low dilutions of the template DNA, either from plasmid or naturally infected tissue. As expected, plasmid template performs best, as lower concentrations can be reliably detected at higher Ct values, but the resulting Ct cut-off is not significantly different from the Ct cut-off recorded using infected tissue.

A Ct of 39 is currently used as the cut-off for positive results, and our experiments indicate both of these assays are within the limits of detection at this Ct. The combined CFP and WCC duplex assay does have a slightly reduced cut-off Ct of 38.1 for CFP and 37.5 for WCC, meaning CFP and WCC DNA cannot be reliably detected above this Ct in the duplex assay. The validation experiments confirmed the limit of detection for both CFP and WCC occurs at a Ct of 39.5 and 40.5, respectively, when plasmid template is used as positive control material. These Ct values do not change significantly when naturally occurring infected WCC tissue is used as template with Cts of 39.3 and 39.4, respectively. This is in agreement with the CFP detection method as published by Vrålstad et al., 2009 which gave a positive cut-off Ct of 39. All future reports of CFP and WCC positive results will use these cut-off Ct values. Where a Ct value falls between these new cut-off limits and 39 they will be reported as "suspect" positive results.

Table 6: Limit of detection and reproducibility of CFP and WCC singleplex qPCR assays and CFP and WCC combined duplex qPCR assay.

qPCR to detect	Template type	Lowest dilution	Mean Ct (20)	StDev	Ct Cut-off
<b>Crayfish Plague</b>	Plasmid alone	10 -8 (1:2)	39.25	0.202	39.5
	CFP-Infected WCC tissue	10 -4 (1:2)	39.00	0.325	39.3
	Plasmid spiked filters	10 -4 (1:2)	38.36	0.282	38.6
<b>WCC</b>	Plasmid alone	10 -10 (1:2)	40.10	0.439	40.5
	WCC Tissue	10 -5 (1:2)	39.00	0.433	39.4
<b>CFP and WCC duplex</b>	CFP Infected WCC tissue	10 -4 (1:2)	CFP 37.91	0.232	38.1
	WCC tissue	10 -4 (1:2)	WCC 37.39	0.142	37.5

#### 4.8 False Positive Results

The successful combining of assays to detect CFP and WCC left a space in one of our multiplex qPCR assays to detect NICS. An assay to detect Chinese Mitten Crab (CMC) *Eriocheir sinensis* was selected as a possible replacement. The assay was tested using positive control material (CMC tissue; GMIT) and worked satisfactorily both in singleplex and multiplex reactions, with no apparent issues. All 2020 CFP-positive eDNA samples were then tested for the presence of NICS (and now CMC) as normal. CMC was detected in all samples with Ct values ranging from 30-36, so well within the cut-off limits for a positive detection.

CMC presence at 20 separate sites spread across Ireland was not a credible result. There are three records on the Biodiversity Ireland website of CMC sightings in Ireland, however, only one of these records has been verified, therefore, the only substantial evidence for the presence of CMC in Ireland is limited to Waterford Harbour. As it is prohibitively difficult to sequence the end product of a qPCR reaction, we needed a suitable conventional PCR test for our samples, which could then be sequenced. During this programme, interrogation of the literature and testing of multiple primer pairs which target the 16S, ITS and COI gene sequences of CMC was completed. Despite the known difficulties associated with cPCR and eDNA, we successfully obtained suitable PCR products for sequencing. All PCR products selected for sequencing had approximately the same fragment size as the positive control material, suggesting we could be amplifying CMC in these PCRs.

Sequencing analysis was hampered by the poor quality of the sequences, with heterogeneity and short fragments present in all samples sent for sequencing. After considerable effort and quality control procedures, a BLAST analysis was possible, which searches the Genbank database for suitable matches to our sequences, and found our CMC specific qPCR was, in fact, detecting amphipod and rotifer species normally and commonly found in freshwater environments. A SYBR green dye-based real time PCR test which uses a melt curve analysis to identify CMC, was also tested. While

our positive control material worked very well in this test, our eDNA samples had multiple melt-curve peaks meaning the data was inconclusive (Figure 5).

In this case, the qPCR to detect CMC was not suitable for use with eDNA samples and will not be used in any future NCPSP testing. While false positive results are possible with eDNA and any qPCR method, we believe the qPCR assays to detect CFP, WCC and the 8 NICS to be substantially more specific and sensitive as they were designed to both specifically detect the pathogen/crayfish in question and also have the ability to discriminate between related species. The CMC assay was designed to detect any crab species that may be present as an allergen in food using non-specific primers to target the 16S rRNA sequence, which is ubiquitously found in all organisms making it unsuitable for use with DNA extracted from environmental samples. A new eDNA assay designed especially for eDNA samples for detection of CMC (Knudsen et al., 2022) will be used in future work.

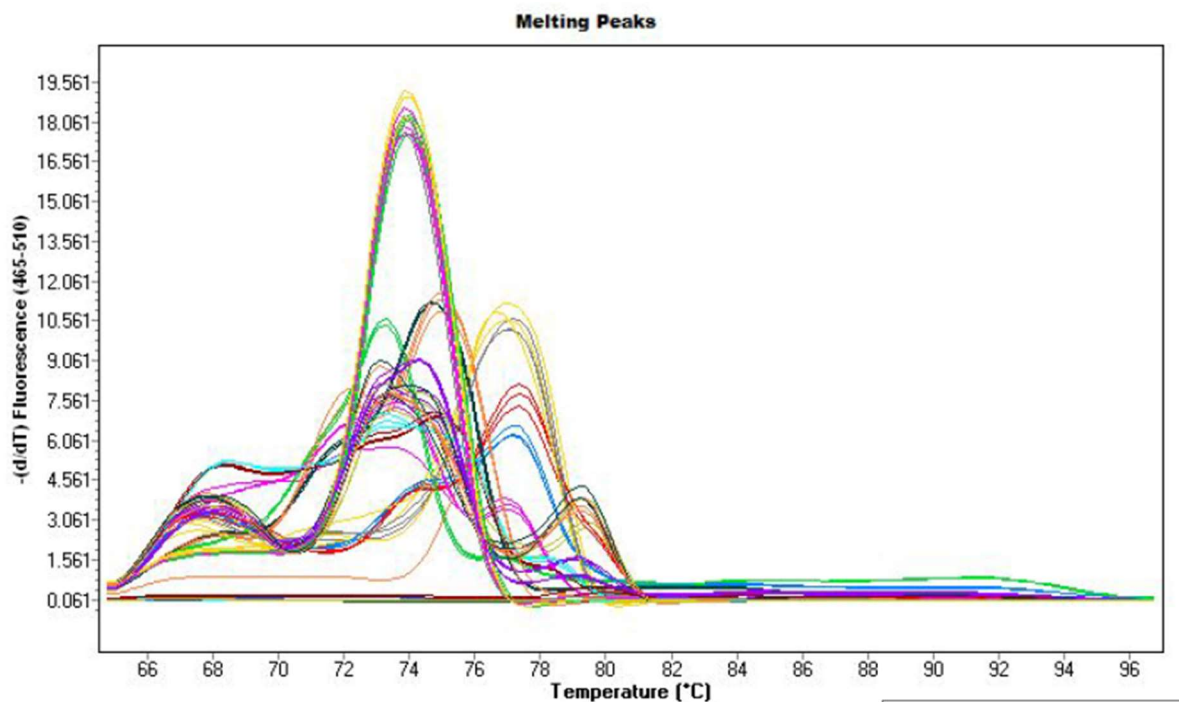


Figure 4: Melt curve analysis of CMC positive control material (single peak at ~74C) and eDNA samples which tested positive for the presence of CMC in a SYBR green dye-based qPCR test.

## 5. Significance of the Results

### 5.1 Spread of CFP within/between catchments

At the end of the 2020-2021 NCPSP, there is further evidence for the continued spread of CFP both within and between catchments. New sites in previously identified CFP-positive catchments have been confirmed positive, and three additional catchments, Sligo, Moy, and Shannon 26C, are now confirmed by eDNA as CFP-positive for the first time. See Figure 1. We failed to detect CFP in Shannon 26A in this programme following a positive confirmation by eDNA at one site in 2019. Shannon 25A is also now confirmed CFP-positive from mortality data which represents a total of 16 affected catchments.

The genotyping results tell us there are 3 separate CFP subtypes present in Ireland. This would suggest 3 separate introduction events have occurred to bring these subtypes to Irish waterways, however, we cannot determine that for certain with the current data. The B/C genotype remains confined to the Erne catchment, but the A and D genotypes are now spread across the island with both A and D genotypes and are recorded as present in the Corrib, Shannon 26D, Sligo and Moy catchments. The most likely explanation for the continued spread between catchments is human activity, such as angling, kayaking and potentially also the sampling activities of other state agencies or environmental consultancy firms with a lack of proper or any biosecurity measures when moving from one river system to another. It is imperative the Check Clean Dry biosecurity message is delivered effectively to curb the spread of the disease.

## 5.2 Status of WCC and non-detection of NICS

WCC were detected in every catchment sampled with the exception of two catchments, Shannon 25D and 26C. This is in line with the results of the 2018-2019 programme, which also failed to detect WCC in both of those catchments. The field survey data correlated extremely well with our available eDNA results, and while this work is preliminary, it would suggest the results of the eDNA sampling accurately reflects the WCC status in each catchment. The status of the WCC in these catchments and also the Barrow and Suir remains a concern, but overall there seems to be an improvement in WCC density in most catchments since 2018. A comparison of the available data from 2018 with the data from 2020 is shown in figure 5.

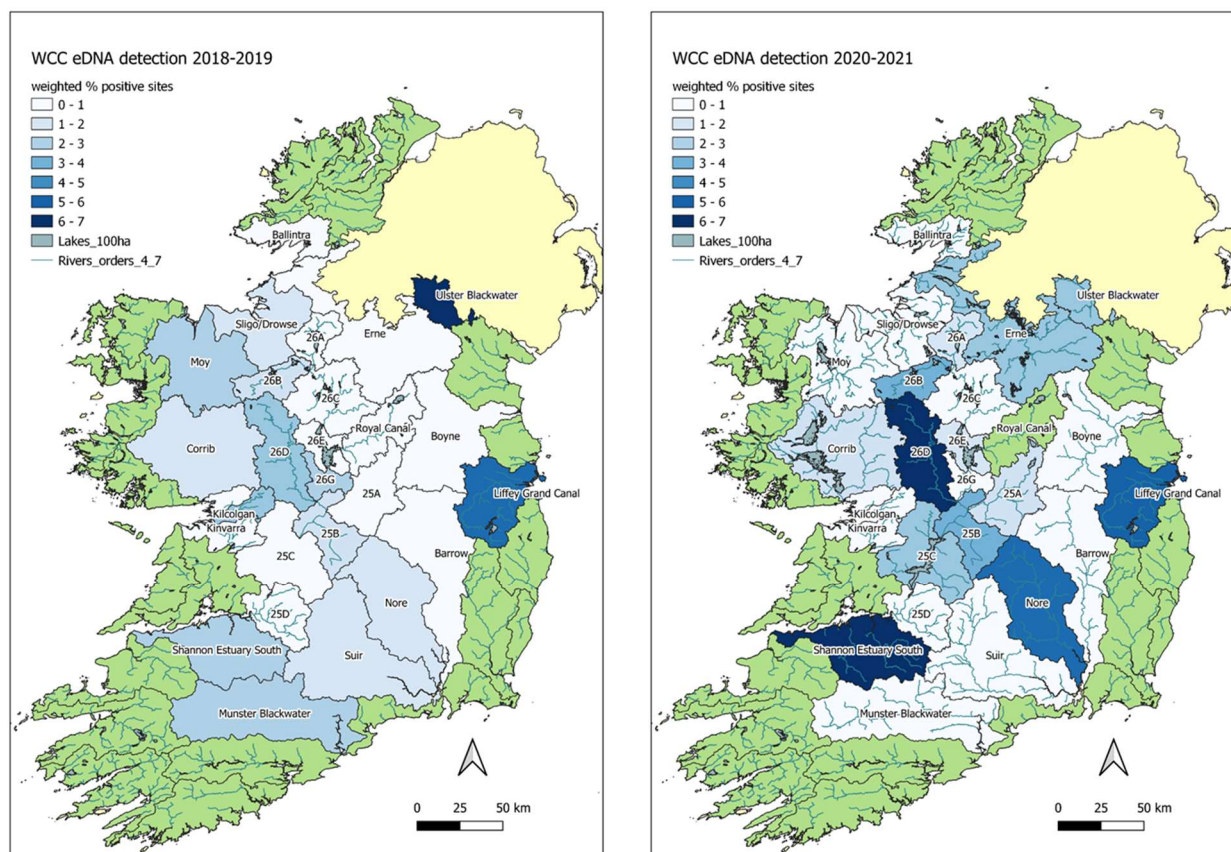


Figure 5: Maps of 2018-2019 and 2020-2021 sampled catchments with positive WCC results indicated. Colour gradient is calculated using the % of positive sites in that catchments with darker blue colour, indicating a higher percentage of positive sites. A weighted calculation was used to account for the varying number of sites in each catchment.

Our multiplex assays, to detect any of the 8 crayfish species which may have acted as disease vectors in the introduction of CFP to Ireland, did not detect the presence of any NICS DNA in our CFP-positive catchments. Preliminary validation results using plasmid DNA as a quantifiable starting template for one of these assays has shown these tests to be sensitive and fit for purpose with the cut-off Ct within the limits of detection. Full validation of these assays is yet to be completed but initial results indicate the sensitivity and specificity of this method is comparable with the assay to detect CFP and WCC, which have been validated and are suitable for use in this work.

## 5.3 Negative results

Sites changing from positive to negative and vice versa for both CFP and WCC detections in the results of the time series experiment illustrate the potential impact of sampling methodology and external factors on our results. In general, there was an increase in the total number of CFP and WCC detections during the November sampling times. This may be due to a substantially increased water volume and flow in the rivers with more debris observed also (due to increased rain and storm-like conditions), which potentially increases the amount of nucleic acid circulating in the water and the

amount available for detection. In particular, the fact we can detect circulating spores from crayfish plague may explain the difference in CFP detections compared to WCC during the time series sampling. Additional biological and environmental factors should also be considered, the moulting and reproductive aspects of the crayfish lifecycle, water temperature or turbidity may impact our ability to detect WCC or CFP at any particular time point.

## 6 Discussion Points and Future Work

### 6.1 Project improvement and refinement

While the two surveillance programmes were clearly successful overall in their aims and objectives, there is still scope for refinement and improvement. A future, more representative sampling programme should address issues such as sampling frequency, seasonal sampling, total site numbers and the impact of environmental conditions. eDNA as a surveillance tool is demonstrably useful and also arguably more efficient than field ecology studies, however, there are still unanswered questions regarding the conclusions that can be drawn from the data generated, particularly for negative results. A positive or negative result represents a snap shot in time, we can only infer the presence/absence of the organism at that particular time, in that particular location, under those particular environmental conditions. In the case of negative results, single point eDNA data cannot be used as conclusive evidence for the absence of plague or crayfish, however, this uncertainty can be significantly reduced by repeat sampling either over time and or by sampling additional locations. The better the resolution of any sampling regime, the less chance there is of failing to detect the presence of plague or crayfish.

The time series results for the Barrow catchment illustrate this point well, we failed to detect either CFP or WCC during 2021, which was an unexpected outcome considering the history of the catchment. Repeat sampling of these sites is necessary, and overall it may be more useful to describe the results and analysis in terms of trending rather than end point data. While an initial 2-year schedule for the first two programmes was a reasonable format, to begin with, a longer time period may be more suitable to accommodate trending analyses and facilitate protocol improvements in future programmes.

### 6.2 Future Work

The results from the time series experiment did show a significant change in the number of positive and negative detections for both CFP and WCC during July and November sampling time points in the Shannon Estuary South catchment. Future surveillance programmes should consider, where possible, shifting the sampling window towards November to maximise the chances of positive detections. A Spring sampling time point (Mar-April) has not been tested to date and may be worth investigating further in future work.

The use of passive surveillance techniques for eDNA have been shown to be very effective in some similar studies (Bessey et al., 2021; Tsuji et al., 2019), and it is worth trialling this option on a pilot study scale in future surveillance programmes. Passive surveillance would monitor the eDNA passing through a particular site over a number of hours compared to the relatively short period of time currently used to actively filter 3 x 5 litres of water using a pump. Testing of this sampling method requires a planned and monitored study and a direct comparison with the current active filtration methods employed. The use of a passive rather than active sampling protocol would provide significant logistical benefit for future programmes considering the high resource input required for active sampling.

A new Memorandum of Understanding between National Parks and Wildlife Service and the Marine Institute was agreed in 2022 to continue the work of the NCPSP for an extended period of four years.

### 6.3 Translatable research

In terms of using the data generated to pinpoint the location of WCC habitats or estimate WCC population densities, a number of additional, complimentary and concurrent experiments are required. In order to gain a better insight into the relationship between qPCR Ct values from a water sample and the real world numbers of crayfish in the immediate and surrounding areas, an experiment with caged animals in a suitable location would be necessary, alongside concurrent field ecology and molecular detection experiments. As WCC are protected under Irish and European legislation, there are inherent difficulties with such experiments, and careful consideration would be necessary from an animal welfare point of view. The combined field ecology and molecular detection studies completed as part of this surveillance programme cannot give a definitive explanation of the relationship between molecular detections and real-world crayfish population densities, however, it could provide baseline evidence of comparable and similar results with some indication that the molecular detection method is actually more sensitive than a traditional field ecology approach. There is also the added advantage of being able to detect historical DNA signatures, with historical in the case of water samples meaning days or weeks old, so still relevant in the context of real-time assessment of WCC population densities.

This document has been drafted as an internal report for NPWS. All information herein will be submitted for peer-reviewed publication.

## 7 Acknowledgments

We would like to acknowledge the efforts of all NPWS and IFI staff whom enabled the completion of our field sampling program

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## Appendix I

Results of the CFP and WCC tests at all sites in all catchments 2020-2021.

Catchment	Site Name	Sample Ref	Date	Lat	Long	River/Lake	CFP#	Mean Ct	WCC#	Mean Ct
Shannon Estuary South	Castlemahon Bridge	E 3 20 S1	30/06/2020	52.4304	-9.0103	Deel	negative		negative	
Shannon Estuary South	Grange Bridge	E 3 20 S2	30/06/2020	52.4628	-9.0188	Deel	negative		positive	37.59
Shannon Estuary South	Rathkeale	E 3 20 S3	30/06/2020	52.5195	-8.9503	Deel	negative		positive	40.10
Shannon Estuary South	Kilcool Bridge	E 3 20 S4	30/06/2020	52.5399	-8.9784	Deel	negative		negative	
Shannon Estuary South	Askeaton Main Street	E 3 20 S5	30/06/2020	52.6008	-8.9739	Deel	positive	36.06	positive	40.38
Shannon Estuary South	River Loobagh	E 3 20 S6	01/07/2020	52.3938	-8.6439	Loobagh	negative		positive	29.80
Shannon Estuary South	Bruree	E 3 20 S7	01/07/2020	52.4236	-8.6613	Maigue	negative		positive	29.00
Shannon Estuary South	Athlacca (Howardstown)	E 3 20 S8	01/07/2020	52.4518	-8.6721	Morning Star	positive	26.73	positive	26.75
Shannon Estuary South	River Camogue, Manister	E 3 20 S9	01/07/2020	52.5179	-8.6658	Camogue	positive	27.06	positive	27.48
Shannon Estuary South	Croom	E 3 20 S10	01/07/2020	52.5189	-8.7183	Maigue	positive	33.80	positive	34.98
Shannon Estuary South	Castleroberts Bridge	E 3 20 S11	01/07/2020	52.5433	-8.7673	Maigue	positive	35.08	positive	37.78
Shannon Estuary South	Adare	E 3 20 S12	01/07/2020	52.5687	-8.7839	Maigue	negative	35.81	negative	39.59
Shannon Estuary South	Castlemahon Bridge	E 18 20 S1	11/11/2020	52.43024	-9.01055	Deel	negative		negative	
Shannon Estuary South	Grange Bridge	E 18 20 S2	11/11/2020	52.46271	-9.01882	Deel	negative		positive	39.45
Shannon Estuary South	Rathkeale	E 18 20 S3	11/11/2020	52.51966	-8.95020	Deel	negative		positive	37.82
Shannon Estuary South	Kilcool Bridge	E 18 20 S4	11/11/2020	52.54112	-8.97672	Deel	negative	39.51	negative	
Shannon Estuary South	Askeaton Main Street	E 18 20 S5	13/11/2020	52.60083	-8.97398	Deel	positive	37.27	negative	38.01
Shannon Estuary South	River Loobagh	E 18 20 S6	12/11/2020	52.39724	-8.66161	Loobagh	positive	37.38	positive	33.11
Shannon Estuary South	Bruree	E 18 20 S7	12/11/2020	52.42356	-8.66107	Maigue	positive	35.17	positive	35.75
Shannon Estuary South	Athlacca (Howardstown)	E 18 20 S8	12/11/2020	52.45174	-8.67200	Morning Star	positive	32.63	positive	36.26
Shannon Estuary South	River Camogue, Manister	E 18 20 S9	12/11/2020	52.51785	-8.66588	Camogue	positive	29.71	positive	35.58
Shannon Estuary South	Croom	E 18 20 S10	13/11/2020	52.51892	-8.71830	Maigue	positive	31.38	positive	35.56
Shannon Estuary South	Castleroberts Bridge	E 18 20 S11	13/11/2020	52.54331	-8.76760	Maigue	positive	31.39	positive	36.94
Shannon Estuary South	Adare	E 18 20 S12	13/11/2020	52.56870	-8.78390	Maigue	positive	32.83	positive	37.38
Shannon Estuary South	Castlemahon Bridge	E 1 21 S1	29/06/2021	52.4304	-9.0103	Deel	negative		negative	
Shannon Estuary South	Grange Bridge	E 1 21 S2	29/06/2021	52.4628	-9.0188	Deel	negative		negative	

Shannon Estuary South	Rathkeale	E 1 21 S3	29/06/2021	52.5195	-8.9503	Deel	negative		negative	
Shannon Estuary South	Kilcool Bridge	E 1 21 S4	29/06/2021	52.5399	-8.9784	Deel	negative		negative	
Shannon Estuary South	Askeaton Main Street	E 1 21 S5	29/06/2021	52.6008	-8.9739	Deel	negative		negative	
Shannon Estuary South	River Loobagh	E 1 21 S6	30/06/2021	52.3938	-8.6439	Loobagh	positive	38.94	positive	34.47
Shannon Estuary South	Bruree	E 1 21 S7	30/06/2021	52.4236	-8.6613	Maigue	negative		positive	35.59
Shannon Estuary South	Athlacca (Howardstown)	E 1 21 S8	30/06/2021	52.4518	-8.6721	Morning Star	negative		negative	
Shannon Estuary South	River Camogue, Manister	E 1 21 S9	30/06/2021	52.5179	-8.6658	Camogue	negative		negative	
Shannon Estuary South	Croom	E 1 21 S10	30/06/2021	52.5189	-8.7183	Maigue	negative		negative	
Shannon Estuary South	Castleroberts Bridge	E 1 21 S11	30/06/2021	52.5433	-8.7673	Maigue	negative		negative	
Shannon Estuary South	Adare	E 1 21 S12	30/06/2021	52.5687	-8.7839	Maigue	negative		negative	
Shannon Estuary South	Ballinahinch Bridge	E 3 21*	09/07/2021	52.40913	-8.43936	Morning Star	positive	30.18	positive	31.65
Shannon Estuary South	Castlemahon Bridge	E 18 21 S1	11/11/2021	52.43024	-9.01055	Deel	negative		negative	
Shannon Estuary South	Grange Bridge	E 18 21 S2	11/11/2021	52.46271	-9.01882	Deel	negative		positive	36.97
Shannon Estuary South	Rathkeale	E 18 21 S3	11/11/2021	52.51966	-8.95020	Deel	negative		positive	36.85
Shannon Estuary South	Kilcool Bridge	E 18 21 S4	11/11/2021	52.54112	-8.97672	Deel	negative		negative	
Shannon Estuary South	Askeaton Main Street	E 18 21 S5	11/11/2021	52.60083	-8.97398	Deel	negative		negative	
Shannon Estuary South	River Loobagh	E 18 21 S6	10/11/2021	52.39724	-8.66161	Loobagh	positive	34.30	positive	34.29
Shannon Estuary South	Bruree	E 18 21 S7	10/11/2021	52.42356	-8.66107	Maigue	positive	36.12	positive	36.24
Shannon Estuary South	Athlacca (Howardstown)	E 18 21 S8	10/11/2021	52.45174	-8.67200	Morning Star	negative		negative	
Shannon Estuary South	River Camogue, Manister	E 18 21 S9	10/11/2021	52.51785	-8.66588	Camogue	negative		negative	
Shannon Estuary South	Croom	E 18 21 S10	10/11/2021	52.51892	-8.71830	Maigue	positive	38.39	positive	37.22
Shannon Estuary South	Castleroberts Bridge	E 18 21 S11	10/11/2021	52.54331	-8.76760	Maigue	negative		negative	
Shannon Estuary South	Adare	E 18 21 S12	10/11/2021	52.56870	-8.78390	Maigue	positive	37.00	positive	37.19
Barrow	Two Mile Bridge	E 4 20 S1	07/07/2020	53.15546	-7.3692	Barrow	positive	35.98	negative	36.10
Barrow	Owenass	E 4 20 S2	07/07/2020	53.09624	-7.37968	Owenass	positive	30.89	positive	33.78
Barrow	Millgrove	E 4 20 S3	07/07/2020	53.21849	-7.10309	Figile	negative		negative	
Barrow	Rathangan	E 4 20 S4	07/07/2020	53.22075	-6.99262	Slate	negative		negative	38.59
Barrow	Monasterevin	E 4 20 S5	08/07/2020	53.13728	-7.06422	Barrow	negative		negative	
Barrow	Athy	E 4 20 S6	08/07/2020	52.99178	-6.9843	Barrow	negative		negative	
Barrow	Tankerstown Bridge	E 4 20 S7	08/07/2020	52.94038	-6.95414	Barrow	negative		negative	
Barrow	Carlow Town	E 4 20 S8	09/07/2020	52.83836	-6.93435	Barrow	negative		negative	

Barrow	Leighlinbridge	E 4 20 S9	09/07/2020	52.73619	-6.97886	Barrow	negative		negative	
Barrow	Royal Oak Bridge	E 4 20 S10	09/07/2020	52.69999	-6.98059	Barrow	negative		negative	
Barrow	Gorse Bridge	E 4 20 S11	09/07/2020	52.63009	-6.98999	Barrow	negative		negative	
Barrow	Graignamanagh	E 4 20 S12	09/07/2020	52.5391	-6.9539	Barrow	negative		negative	
Barrow	Two Mile Bridge	E 19 20 S1	16/11/2020	53.15546	-7.3692	Barrow	positive	35.93	negative	36.60
Barrow	Owenass	E 19 20 S2	16/11/2020	53.09624	-7.37968	Owenass	negative		negative	
Barrow	Millgrove	E 19 20 S3	16/11/2020	53.21849	-7.10309	Figile	positive	36.98	positive	37.60
Barrow	Rathangan	E 19 20 S4	16/11/2020	53.22075	-6.99262	Slate	negative		negative	36.70
Barrow	Monasterevin	E 19 20 S5	17/11/2020	53.13728	-7.06422	Barrow	positive	36.23	positive	38.11
Barrow	Athy	E 19 20 S6	17/11/2020	52.99178	-6.9843	Barrow	negative		negative	
Barrow	Tankerstown Bridge	E 19 20 S7	17/11/2020	52.94038	-6.95414	Barrow	negative	41.10	negative	
Barrow	Carlow Town	E 19 20 S8	17/11/2020	52.83836	-6.93435	Barrow	negative		negative	
Barrow	Leighlinbridge	E 19 20 S9	17/11/2020	52.73619	-6.97886	Barrow	positive	36.20	negative	
Barrow	Royal Oak Bridge	E 19 20 S10	18/11/2020	52.69999	-6.98059	Barrow	negative	40.79	negative	
Barrow	Gorse Bridge	E 19 20 S11	18/11/2020	52.63009	-6.98999	Barrow	negative	40.04	negative	
Barrow	Graignamanagh	E 19 20 S12	18/11/2020	52.5391	-6.9539	Barrow	negative		positive	38.22
Barrow	Two Mile Bridge	E 2 21 S1	07/07/2021	53.15546	-7.3692	Barrow	negative		negative	
Barrow	Owenass	E 2 21 S2	07/07/2021	53.09624	-7.37968	Owenass	negative		negative	
Barrow	Millgrove	E 2 21 S3	07/07/2021	53.21849	-7.10309	Figile	negative		negative	
Barrow	Rathangan	E 2 21 S4	07/07/2021	53.22075	-6.99262	Slate	negative		negative	
Barrow	Monasterevin	E 2 21 S5	07/07/2021	53.13728	-7.06422	Barrow	negative		negative	
Barrow	Athy	E 2 21 S6	07/07/2021	52.99178	-6.9843	Barrow	negative		negative	
Barrow	Tankerstown Bridge	E 2 21 S7	08/07/2021	52.94038	-6.95414	Barrow	negative		negative	
Barrow	Carlow Town	E 2 21 S8	08/07/2021	52.83836	-6.93435	Barrow	negative		negative	
Barrow	Leighlinbridge	E 2 21 S9	08/07/2021	52.73619	-6.97886	Barrow	negative		negative	
Barrow	Royal Oak Bridge	E 2 21 S10	08/07/2021	52.69999	-6.98059	Barrow	negative		negative	
Barrow	Gorse Bridge	E 2 21 S11	08/07/2021	52.63009	-6.98999	Barrow	negative		negative	
Barrow	Graignamanagh	E 2 21 S12	08/07/2021	52.5391	-6.9539	Barrow	negative		negative	
Barrow	Two Mile Bridge	E 19 21 S1	15/11/2021	53.15546	-7.3692	Barrow	negative		negative	
Barrow	Owenass	E 19 21 S2	15/11/2021	53.09624	-7.37968	Owenass	negative		negative	
Barrow	Millgrove	E 19 21 S3	15/11/2021	53.21849	-7.10309	Figile	negative		negative	

Barrow	Rathangan	E 19 21 S4	15/11/2021	53.22075	-6.99262	Slate	negative		negative	
Barrow	Monasterevin	E 19 21 S5	15/11/2021	53.13728	-7.06422	Barrow	negative		negative	
Barrow	Athy	E 19 21 S6	16/11/2021	52.99178	-6.9843	Barrow	negative		negative	
Barrow	Tankerstown Bridge	E 19 21 S7	16/11/2021	52.94038	-6.95414	Barrow	negative		negative	
Barrow	Carlow Town	E 19 21 S8	16/11/2021	52.83836	-6.93435	Barrow	negative		negative	
Barrow	Leighlinbridge	E 19 21 S9	16/11/2021	52.73619	-6.97886	Barrow	negative		negative	
Barrow	Royal Oak Bridge	E 19 21 S10	16/11/2021	52.69999	-6.98059	Barrow	negative		negative	
Barrow	Gorse Bridge	E 19 21 S11	17/11/2021	52.63009	-6.98999	Barrow	negative		negative	
Barrow	Graignamanagh	E 19 21 S12	17/11/2021	52.53939	-6.95384	Barrow	negative		negative	
Nore	Newbridge Cloncough	E 5 20 S1	22/07/2020	52.94200	-7.44420	Nore	positive	37.94	positive	35.70
Nore	Newtown Mill	E 5 20 S2	22/07/2020	52.84630	-7.46130	Goul	negative	39.72	positive	36.11
Nore	Tallyho Bridge	E 5 20 S3	22/07/2020	52.83580	-7.37270	Nore	negative	39.95	negative	37.34
Nore	Three Castles bridge	E 5 20 S4	23/07/2020	52.71360	-7.32280	Nore	positive	38.57	positive	36.29
Nore	Jeninstown Park	E 5 20 S5	23/07/2020	52.71489	-7.29203	Dinin	positive	37.19	positive	36.13
Nore	Irishtown	E 5 20 S6	23/07/2020	52.65565	-7.25521	Breagagh	negative		negative	
Nore	Ossory Bridge	E 5 20 S7	23/07/2020	52.64366	-7.22390	Nore	negative		positive	37.78
Nore	Lismalin Bridge	E 5 20 S8	23/07/2020	52.55190	-7.53240	Kings River	negative		positive	35.38
Nore	Bridge SW of Kilbraghan	E 5 20 S9	23/07/2020	52.58740	-7.46780	Munster	negative		positive	36.82
Nore	Thomastown Bridge	E 5 20 S10	24/07/2020	52.52450	-7.13810	Nore	negative		positive	37.35
Nore	Brownsbarn Bridge	E 5 20 S11	24/07/2020	52.50083	-7.09161	Nore	negative		negative	
Suir	Newtown	E 6 20 S1	29/07/2020	52.61620	-7.8964	Suir	negative		negative	37.63
Suir	Camus Bridge	E 6 20 S2	29/07/2020	52.63980	-7.9323	Suir	negative		negative	
Suir	Ballygriffin	E 6 20 S3	29/07/2020	52.51530	-7.9917	Multeen	positive	36.19	negative	
Suir	Cahir Bridge	E 6 20 S4	30/07/2020	52.37490	-7.9267	Suir	negative		negative	37.70
Suir	Ardfinnan	E 6 20 S5	30/07/2020	52.31010	-7.8795	Suir	negative		negative	
Suir	Clonmel Blueway	E 6 20 S6	30/07/2020	52.35230	-7.693	Suir	negative		positive	36.83
Suir	Twomilebridge	E 6 20 S7	30/07/2020	52.36020	-7.6417	Anner	negative		negative	37.86
Suir	Parkbeg	E 6 20 S8	30/07/2020	52.35510	-7.5167	Carrowclough	negative		positive	37.60
Suir	Carrick on Suir	E 6 20 S9	31/07/2020	52.34420	-7.4109	Suir	negative		negative	
Suir	Fiddown	E 6 20 S10	31/07/2020	52.32970	-7.3173	Suir	negative		negative	
Corrib	Cloonfad	E 7 20 S1	05/08/2020	53.68700	-8.75210	Cloonfad	negative		positive	31.77

Corrib	Castlemargaret Bridge	E 7 20 S2	05/08/2020	53.69699	-8.96624	Robe River	negative		positive	29.40
Corrib	Ballinrobe	E 7 20 S3	05/08/2020	53.62236	-9.22542	Robe River	negative		positive	28.74
Corrib	Cong	E 7 20 S4	05/08/2020	53.54080	-9.28929	Cong	negative		negative	
Corrib	Bridge South of Tuam	E 7 20 S5	05/08/2020	53.49177	-8.88521	Clare	negative		positive	35.63
Corrib	Annaghdown Pier	E 7 20 S6	06/08/2020	53.38680	-9.07770	Lough Corrib	negative		negative	
Corrib	D/S Corrofin	E 7 20 S7	06/08/2020	53.41060	-8.87980	Clare	positive	36.70	positive	35.39
Corrib	Crusheen	E 7 20 S8	06/08/2020	53.34340	-8.90580	Clare	negative		negative	28.86
Corrib	Claregalway	E 7 20 S9	06/08/2020	53.34560	-8.94250	Clare	negative		negative	36.48
Corrib	Curraghline	E 7 20 S10	07/08/2020	53.34184	-9.01936	Clare	negative		negative	37.11
Shannon 26G	Tang	E 8 20 S1	12/08/2020	53.545	-7.8409	Lough Ree	negative		negative	
Shannon 26G	Riverview House	E 8 20 S2	12/08/2020	53.41227	-7.98435	Cross River	negative		negative	37.22
Shannon 26G	Castledaly	E 8 20 S3	12/08/2020	53.3735	-7.7795	Boor	negative		positive	38.75
Shannon 26G	Moydrum	E 8 20 S4	12/08/2020	53.4181	-7.8648	Al	negative		positive	37.53
Shannon 26G	Garrycastle	E 8 20 S5	13/08/2020	53.4183	-7.8972	AL	negative		negative	37.94
Shannon 26G	River Al	E 8 20 S6	13/08/2020	53.41452	-7.90026	Al	negative		positive	40.16
Shannon 26G	Golden Island	E 8 20 S7	13/08/2020	53.4193	-7.9289	AL	negative		positive	36.70
Shannon 25C	Ahanageely Bridge	E 9 20 S1	17/08/2020	53.2055	-8.29061	Killronan	negative		positive	35.79
Shannon 25C	Kilcrow River	E 9 20 S2	17/08/2020	53.1171	-8.3076	Kilcrow	negative		positive	37.20
Shannon 25C	Portumna Bridge	E 9 20 S3	17/08/2020	53.09114	8.1965034	Shannon	negative		negative	
Shannon 25C	Lorrha Village A	E 9 20 S4A	18/08/2020	53.0912	-8.1182	Lorrha stream	negative		positive	37.38
Shannon 25C	Lorrha Village B	E 9 20 S4B	18/08/2020	53.0913	-8.1246	Lorrha stream	negative		positive	35.47
Shannon 25C	Ollatrim Bridge	E 9 20 S5	18/08/2020	52.8667	-7.9996	Ollatrim	negative		positive	35.42
Shannon 25C	Ballyartella Bridge	E 9 20 S6	18/08/2020	52.9018	-8.2397	Nenagh	negative		positive	30.97
Moy	Cloonacannana	E 10 20 S1	26/08/2020	53.96740	-8.93030	Moy	positive	35.82	positive	34.78
Moy	Toormore Bridge	E 10 20 S2	26/08/2020	53.89176	-9.17132	Manulla	negative		positive	31.18
Moy	Barret Street, Ballina	E 10 20 S3	26/08/2020	54.11050	-9.15650	Moy	negative		positive	37.24
Moy	Knockglass Bridge	E 10 20 S4	26/08/2020	54.11480	-9.28890	Deel	negative		positive	30.70
Moy	Upstream POS 2020	E 17 21 1	01/10/2021	54.03657	-8.81681	Moy	positive	34.80	positive	33.30
Moy	Cloonacannona	E 17 21 2	01/10/2021	53.96741	-8.93039	Moy	positive	35.74	positive	35.53
Moy	D/S positive 2020	E 17 21 3	01/10/2021	53.93793	-9.1034	Moy	positive	35.62	positive	35.25
Boyne	Inchamore Bridge	E 11 20 S1	01/09/2020	53.49460	-6.92950	Boyne	negative		positive	35.46

Boyne	Slane Bridge	E 11 20 S2	01/09/2020	53.70361	-6.54135	Boyne	negative		positive	37.38
Boyne	Headfort Bridge	E 11 20 S3	01/09/2020	53.72800	-6.84640	Kells Blackwater	negative		negative	
Boyne	Moynalty River	E 11 20 S4	01/09/2020	53.73780	-6.80200	Moynalty	negative		positive	37.70
Shannon 25A	Lough Owel 1A	E 12 20 S1A	08/09/2020	53.57540	-7.36750	L.Owel	negative		positive	34.83
Shannon 25A	Lough Owel 1B	E 12 20 S1B	08/09/2020	53.55630	-7.36590	L.Owel	negative		negative	37.49
Shannon 25A	Lough Owel 1C	E 12 20 S1C	18/09/2020	53.55358	-7.36042	Royal Canal stream	negative		positive	34.71
Shannon 25A	Kilbeggan	E 12 20 S2	17/09/2020	53.36891	-7.50208	Brosna	negative		positive	38.44
Shannon 25A	Ballyboughlin Bridge, Clara	E 12 20 S3	17/09/2020	53.35394	-7.6446	Gageborough River	negative		positive	35.46
Shannon 25A	Lumcloon NS	E 12 20 S4	17/09/2020	53.21973	-7.797871	Silver River	negative		positive	35.01
Sligo	Gurteen	E 13 20 S1	10/09/2020	53.99980	-8.5031	Owenmore	positive	34.80	positive	33.35
Sligo	Dromahair	E 13 20 S2	10/09/2020	54.23319	-8.30913	Bonet River	negative		positive	33.51
Sligo	Collooney	E 13 20 S3	10/09/2020	54.18741	-8.48393	Owenmore	negative		positive	35.44
Sligo	Ballysadare	E 13 20 S4	11/09/2020	54.20915	-8.50954	Ballysadare River	negative		positive	37.71
Sligo	Boyle Road, Gurteen	E 16 21 2	24/09/2021	53.98731	-8.48662	Owenmore	positive	30.95	positive	35.88
Sligo	Gurteen	E 16 21 1	24/09/2021	53.99971	-8.50383	Owenmore	positive	29.97	positive	32.63
Sligo	Gurteen 2	E 16 21 3	24/09/2021	54.01165	-8.49266	Owenmore	positive	31.73	positive	32.03
Ballintra	Lough Rath	E 14 20 S1	15/09/2020	54.5682	-8.0425	Ballintra	negative		positive	29.84
Ballintra	Aghadullagh	E 14 20 S2	15/09/2020	54.575	-8.1179	Ballintra	negative		positive	30.67
Ballintra	Shannagh Bridge	E 14 20 S3	15/09/2020	54.58961	-8.04992	Bridgetown	negative		negative	
Ballintra	Murvagh	E 14 20 S4	15/09/2020	54.5939	-8.1467	Bridgetown	negative		positive	30.88
Munster Blackwater	Buttevant	E 15 20 S1	24/09/2020	52.23409	-8.66801	Awbeg River	negative		negative	
Munster Blackwater	Castletownroche	E 15 20 S2	24/09/2020	52.17281	-8.46031	Awbeg River	negative		positive	35.07
Munster Blackwater	Kent Bridge, Fermoy	E 15 20 S3	24/09/2020	52.13948	-8.27523	Blackwater River	negative		positive	35.27
Munster Blackwater	Cappaquinn	E 15 20 S4	24/09/2020	52.15031	-7.87146	Blackwater River	negative		negative	
Erne	Lough Gowna	E 16 20 S1	29/09/2020	53.81907	-7.55046	L. Gowna	negative		negative	
Erne	Br 3km SW of Crosskeys	E 16 20 S2	29/09/2020	53.90389	-7.29452	Erne	negative		negative	
Erne	Bruskey Bridge at Carrigan	E 16 20 S3	29/09/2020	53.88649	-7.4046	Erne	negative		negative	
Erne	Legwee	E 16 20 S4	29/09/2020	53.88043	-7.428321	Erne	negative		negative	
Erne	Killydoon	E 16 20 S5	06/10/2020	53.87373	-7.43773	Erne	negative		negative	
Erne	Ballinagh	E 16 20 S6	06/10/2020	53.9101	-7.484	Erne	negative		negative	
Erne	Lisdam, Cavan Town	E 16 20 S7	06/10/2020	54.00402	-7.36671	Cavan River	negative		positive	35.84

Erne	Ballynascarva Br	E 16 20 S8	06/10/2020	54.0927	-7.01309	Dromore	negative		negative	
Erne	Br SE of Fort William, Cootehill	E 16 20 S9	06/10/2020	54.06202	-7.09518	Annalee River	negative		positive	34.87
Erne	Lough Veenagreane	E 16 20 S10	13/10/2020	54.61408	-7.72292	Lough Veenagreane	negative		positive	36.33
Erne	Lough Nageage	E 16 20 S11	13/10/2020	54.61288	-7.732952	Lough Nageage	negative		negative	
Erne	Lough Naveane	E 16 20 S12	13/10/2020	54.61254	-7.72793	Lough Naveane	negative		negative	
Erne	Stream btw lakes	E 16 20 S13	13/10/2020	54.61018	-7.73574	Stream btw lakes	negative		positive	35.48
Erne	Tullylark bridge	E 16 20 S14	13/10/2020	54.58358	-7.78442	Tenor River	negative		positive	38.67
Erne	Pettigo	E 16 20 S15	13/10/2020	54.55206	-7.83319	Termon River	negative		positive	36.17
Ulster Blackwater	at border before Aughnacloy	E 17 20 S1	09/10/2020	54.4084	-7.002	Blackwater	negative		positive	34.32
Ulster Blackwater	Annaghroe Bridge	E 17 20 S2	09/10/2020	54.33842	-6.875246	Blackwater	negative		positive	35.44
Ulster Blackwater	Bridge N/E of Golden	E 17 20 S3	09/10/2020	54.34047	-7.037072	Mountain Water	negative		positive	34.16
Ulster Blackwater	Emyvale	E 17 20 S4	09/10/2020	54.33244	-6.948292	Mountain Water	negative		positive	32.84
Ulster Blackwater	Scotstown	E 17 20 S5	09/10/2020	54.27876	7.0657901	Blackwater	negative		positive	33.66
Ulster Blackwater	Newmills Bridge	E 17 20 S6	09/10/2020	54.29265	-6.896652	Blackwater	negative		positive	33.78
Shannon 25B	Banagher	E 4 21 1	14/07/2021	53.19285	-7.99214	Shannon	negative		negative	
Shannon 25B	Riverstown	E 4 21 2	14/07/2021	53.08277	-7.92207	Little Brosna	negative		positive	36.71
Shannon 25B	Clareen	E 4 21 3	14/07/2021	53.07231	-7.79076	Fuarown River	negative		positive	35.75
Shannon 25B	Kilcolman	E 4 21 4	14/07/2021	53.04951	-7.87871	Rock River	negative		positive	36.57
Shannon 25D	Blackwater Mills	E 5 21 1	04/08/2021	52.71188	-8.60136	Blackwater	negative		negative	
Shannon 25D	Barringtonbridge	E 5 21 2	04/08/2021	52.64488	-8.47474	Slievenohera River	negative		negative	
Shannon 25D	Mulkear	E 5 21 3	04/08/2021	52.66903	-8.52861	Mulkear River	negative		negative	
Shannon 25D	Mill Road	E 5 21 4	04/08/2021	52.6886	-8.62103	Shannon	negative		negative	
Kilcolgan	Site 1 Ballyfa Bridge	E 6 21 1	05/08/2021	53.29185	-8.51422	Clarín	negative		positive	28.38
Kilcolgan	Site 2 Le Carrow	E 6 21 2	05/08/2021	53.23173	-8.52985	Le Carrow	negative		positive	35.67
Kinvarra	Site 3 Kilchreest	E 6 21 3	05/08/2021	53.15042	-8.70914	Clarín	negative		positive	37.07
Clarín	Site 4 Athenry	E 6 21 4	05/08/2021	53.29943	-8.74435	Clarín	negative		positive	33.09
Kilcolgan	Site 5 Dunkellin Bridge	E 6 21 5	05/08/2021	53.21323	-8.81653	Dunkellin River	negative		negative	
Clarín	Site 6 Clarínbridge	E 6 21 6	05/08/2021	53.22782	-8.88107	Clarín	negative		negative	
Shannon 26 E	Kilnacloghy	E 7 21 1	10/08/2021	53.66779	-8.0303	Keelcurragh	negative		negative	
Shannon 26 E	Newtown	E 7 21 2	10/08/2021	53.60394	-8.12691	Hind River	negative		negative	

Shannon 26 E	Red Bridge	E 7 21 3	10/08/2021	53.54599	-7.82143	Inny River	negative		positive	37.00
Shannon 26 E	Ballykeeran	E 7 21 4	10/08/2021	53.44848	-7.92600	Breensford River	negative		positive	33.56
Grand Canal	Lock no 9	E 9 21 1	17/08/2021	53.32919	-6.39753	Grand Canal	negative		negative	
Grand Canal	Ardclough Bridge	E 9 21 2	18/08/2021	53.29574	-6.56686	Grand Canal	negative		positive	37.18
Grand Canal	Aqueduct over Slaney	E 9 21 3	18/08/2021	53.27707	-6.83775	Grand Canal	negative		negative	
Grand Canal	Monasterevin	E 9 21 4	18/08/2021	53.14282	-7.06947	Grand Canal	negative		negative	
Shannon 26A	Tullynafreave	E 11 21 1	25/08/2021	54.20929	-7.96851	Shannon	negative		positive	34.39
Shannon 26A	Gowra	E 11 21 2	25/08/2021	54.18976	-8.01429	Shannon	negative		positive	34.80
Shannon 26A	Ballyfarnon	E 11 21 3	25/08/2021	54.05821	-8.19475	Feroish	negative		negative	
Shannon 26A	Arigna	E 11 21 4	26/08/2021	54.07718	-8.10805	Arigna River	negative		negative	
Shannon 26A	Bellantra Bridge	E 11 21 5	26/08/2021	54.06079	-8.0597	Shannon	negative		negative	
Shannon 26A	Battlesbridge	E 11 21 6	26/08/2021	53.99633	-8.07844	Shannon	negative		negative	
Shannon 26B	Driney Bridge	E 12 21 1	26/08/2021	53.83472	-8.5981	Anaderryboy (Lung) River	negative		negative	
Shannon 26B	Bridge West of Ballandareen	E 12 21 2	26/08/2021	53.8976	-8.55775	Lung River	positive	35.27	positive	36.64
Shannon 26B	Br ESE of Frenchpark	E 12 21 3	27/08/2021	53.8622	-8.362	Owenaforeesha River	negative	39.62	positive	32.08
Shannon 26B	Cupponagh Bridge	E 12 21 4	27/08/2021	53.95825	-8.40621	Boyle River	negative		negative	
Shannon 26B	Boyle Footbridge	E 12 21 5	27/08/2021	53.97244	-8.30143	Boyle River	positive	33.30	positive	36.03
Shannon 26B	Cootehall	E 12 21 6	27/08/2021	53.98182	-8.16116	Boyle River	negative		negative	
Shannon 26D	Lough Nanannagh stream	E 13 21 1A	31/08/2021	53.74871	-8.97596	Lough Nanannagh Stream	negative		positive	34.99
Shannon 26D	Nannannagh Lough	E 13 21 1B	31/08/2021	53.74931	-8.97817	Nannannagh Lough	negative		positive	34.19
Shannon 26D	Lough O'Flynn	E 13 21 2A	31/08/2021	53.7554	-8.62961	Lough O'Flynn	negative		positive	37.29
Shannon 26D	Lough O'Flynn	E 13 21 2B	31/08/2021	53.76748	-8.62632	Lough O'Flynn	negative		negative	
Shannon 26D	Lough O'Flynn	E 13 21 2C	31/08/2021	53.77255	-8.64679	River Suck	negative		positive	31.72
Shannon 26D	Lough O'Flynn	E 13 21 2D	31/08/2021	53.7715	-8.62273	River Suck	negative		negative	
Shannon 26D	Cloondarra Bridge	E 13 21 3	01/09/2021	53.75094	-8.49937	River Suck	negative		positive	29.55
Shannon 26D	Castlecoote bridge	E 13 21 4	01/09/2021	53.61353	-8.29039	River Suck	negative		negative	
Shannon 26D	Mount Talbot	E 13 21 5	01/09/2021	53.52677	-8.28416	River Suck	negative		positive	36.22
Shannon 26D	Ballyforan	E 13 21 6	01/09/2021	53.46698	-8.27633	River Suck	negative		negative	
Shannon 26D	Ahascragh West Bridge	E 13 21 7	01/09/2021	53.39549	-8.33863	Ahascragh River	negative		negative	
Shannon 26D	Coreen Ford	E 13 21 8	01/09/2021	53.28232	-8.14915	River Suck	negative		negative	
Liffey	Ballymore Eustace Bridge	E 14 21 1	14/09/2021	53.13028	-6.61709	Liffey	negative		positive	35.67

Liffey	Leinster Aquaduct	E 14 21 2	15/09/2021	53.24731	-6.68671	Liffey	negative		positive	32.22
Liffey	Carton House Boathouse	E 14 21 3	15/09/2021	53.38792	-6.56643	Rye water	negative		positive	34.85
Liffey	Leixlip	E 14 21 4	15/09/2021	53.36331	-6.48832	Liffey / Rye Water	negative		positive	36.48
Liffey	Strawberry Beds	E 14 21 5	15/09/2021	53.36204	-6.38526	Liffey	negative		positive	35.82
Shannon 26C	Drumsna	E 15 21 1	16/09/2021	53.92499	-8.01092	Shannon	positive	37.03	negative	
Shannon 26C	Rinn Marina	E 15 21 2	16/09/2021	53.79801	-7.8745	Rinn River	positive	38.01	negative	
Shannon 26C	Ballysaggart Bridge	E 15 21 3	16/09/2021	53.74934	-7.85496	Camlin River	negative		negative	
Shannon 26C	Lanesborough	E 15 21 4	16/09/2021	53.67277	-7.99744	Lough Ree / Shannon	negative		negative	

\*additional sample from mortality site S/16/21

#Must be positive in >1 replicate to be designated a positive site (replicate information not shown)