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Investigation of the first recent crayfish plague outbreak in Ireland and its subsequent spread in the Bruskey River and surrounding areas

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Abstract – White-clawed crayfish (*Austropotamobius pallipes*) is a keystone species found in western European freshwater bodies, where it has suffered drastic declines due to pathogens, competition with non-indigenous crayfish species (NICS) and habitat deterioration. In Ireland, populations of (naturalised) *A. pallipes* have been considered healthy and abundant mainly because no diseases or NICS have been reported in the past decades. The present study investigated a sudden mass mortality event that occurred in the Erne catchment in 2015. Molecular analysis confirmed that the cause of the event was infection by the oomycete *Aphanomyces astaci* (the causative agent of the crayfish plague). While in 2015 the spread of the pathogen appeared to remain confined to the outbreak's epicentre and nearby upstream waters, follow up surveys using conventional methods and environmental DNA (eDNA) approaches indicated that by a year later (2016), the pathogen was still present and had spread downstream beyond Lough Gowna. No NICS were detected during the surveys conducted. This crayfish plague outbreak is of grave concern to Irish white-clawed crayfish and associated ecosystems.

Keywords: *Austropotamobius pallipes* / *Aphanomyces astaci* / crayfish plague / environmental DNA / Ireland

1 Introduction

The White-Clawed Crayfish (*Austropotamobius pallipes*, hereafter WCC) is a freshwater crayfish found in rivers, streams and lakes generally characterised by a calcareous influence. It is one of five crayfish species native to Europe (Kouba *et al.*, 2014) and has undergone drastic declines due to pathogens (Becking *et al.*, 2021; Grandjean *et al.*, 2019), interspecific competition with alien species and habitat deterioration (Holdich and Rogers, 1997; Reynolds, 1998; Souty-Grosset *et al.*, 2006). WCC were likely translocated into Ireland several centuries ago (Gouin *et al.*, 2003), but are now considered naturalised and a keystone species of freshwater systems with protection under the Habitats Directive (92/43/EEC, Annex II, Annex V), the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention, Appendix III), the Wildlife Act (1976, Amendment Act, 2000) and Wildlife (N.I.) Order 1985.

One of the main causes of WCC's declines has been the spread of a lethal pathogen referred to as "the crayfish plague", whose causative agent is the oomycete (water mould) *Aphanomyces astaci,* Schikora 1906 (Bouallegui, 2021). The spread of this pathogen is mostly facilitated by carrier species that are generally unaffected (such as the signal crayfish, *Pacifastacus leniusculus*) (*e.g.* Kaldre *et al.*, 2017), but can transmit the disease to other susceptible species/populations if introduced (Panteleit *et al.*, 2017). The life cycle of *A. astaci* includes free-swimming spores that can persist for

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days or even weeks and hence can pose a risk for alternative pathways such as the use of contaminated wet gear (Strand *et al.*, 2012). The Invasive Species Specialist Group (ISSG) of IUCN has listed *A. astaci* as one of the 100 worst invasive species in the world (Lowe *et al.*, 2004) and this is not only due to its ease of spread, but also to the high incidence of mortality in WCC populations (Martín-Torrijos *et al.*, 2017).

Crayfish plague diagnostic methods originally relied on microbiological isolation of the pathogen from moribund animals followed by re-infection of healthy individuals. However, molecular DNA-based methods have been increasingly adopted thanks to their high sensitivity and specificity. Early methods were based on end-point PCR (Oidtmann *et al.*, 2006, 2002), but lacked specificity (Ballesteros *et al.*, 2007), whereas more recent methods are based on quantitative real-time PCR (qPCR) and target genes that detect the presence of *A. astaci*'s DNA both unambiguously and with high sensitivity (Filipová *et al.*, 2013; Hochwimmer *et al.*, 2009; Vrålstad *et al.*, 2009). The same methods have also been increasingly applied to test not only tissue from infected individuals, but also environmental samples such as water or sediment, in which whole spores or even environmental DNA (eDNA) can be successfully detected (Robinson *et al.*, 2018; Strand *et al.*, 2011).

Irish WCC populations have been relatively unaffected by the crayfish plague mostly because of the absence of any other known carrier species in the island of Ireland, with only one previous crayfish plague outbreak reported in 1980s (Matthews and Reynolds, 1990, 1992; Reynolds, 1998). Since then, no mass mortality of WCC has been reported in Ireland, until 2015 when an outbreak occurred in the Bruskey River (Erne catchment) in County Cavan, which was characterised by signs of crayfish plague (mostly due to lack of evidence of other causes and no other species being affected in the same area). Thus, the present study aimed at (i) elucidating the cause of such a mass mortality event, (ii) assessing the status of the local population post-outbreak and (iii) monitoring the potential spread of the disease in downstream and upstream areas. This was achieved by using genetic methods to confirm the occurrence of *A. astaci*'s DNA in dead and moribund animals as well as in the water column and assessing the presence of dead or live crayfish using conventional methods (visual and trapping surveys).

2 Materials and methods

2.1 Monitoring strategy and survey area

The initial outbreak was reported by locals in Killydoon (GPS coordinates: 53.868245, −7.442526) in a small stretch of the Bruskey River (Erne catchment) in County Cavan (Fig. 1). Targeted visual and hand-net surveys were carried out in the affected areas immediately after the suspected crayfish plague outbreak was reported on 6th July 2015, as well as in July–December 2015. During these surveys dead crayfish (n = 44) were collected and preserved frozen for subsequent analysis. Visual, hand-net, trapping and eDNA surveys were then conducted a year later in August-September 2016 (described below). A follow-up visual and hand-net opportunistic survey was ultimately conducted two years later in October 2017.

For the 2016 survey, a total of 24 sites were selected considering accessibility as well as the complex hydrological features of the areas surrounding the 2015 outbreak epicentre (Tab. 1 and Fig. 1). Between 24th August and 28th September 2016, each site was visited twice, and presence of crayfish was determined using conventional capturing methods, as described below. For each site, hydrochemical properties were measured using a portable HACH HQ40d meter (in the field) and a SHIMADZU 1280 UV-VIS spectrophotometer (in the lab). These included surface water temperature, dissolved oxygen (DO mg/L and % saturation), conductivity, pH and turbidity (absorbance at 540 nm and 750 nm). Additionally, the following environmental parameters were recorded at each site: weather conditions, substrate type, presence of vegetation in the water and in the riparian zone, and approximate water flow (rivers/streams only). Prior to and after visiting each site, appropriate biosecurity measures were adopted (*e.g.* all equipment was decontaminated by immersion in 1% Virkon aquatic and/or 10% sodium hypochlorite solution).

2.2 Crayfish and NICS conventional survey

Under appropriate licensing permits (NPWS Licence number C112/2016), a range of traditional methods were used to detect the presence of crayfish in the surveyed areas. These were modified from the approach described in Reynolds *et al.* (2010) and included five minutes hand and hand-net searches, as well as trapping using baited traps (baits included approximately 50 g of commercial cat food). For the latter, a string of four (Swedish type) rigid funnel traps (four meters apart from each other) was set in each site either overnight (on first visit) or over five nights (on second visit). Crayfish presence and abundance was determined as number of crayfish caught per hand and net search (over five minutes) and Catch Per Unit Effort (CPUE) as number of individuals trapped per day (*i.e.* either 1 day or 5 days). Captured crayfish were counted, measured (carapace length), weighed, sexed and marked (adults only). Markings were performed by clipping the left or right uropod (V-notch) using sterile scissors. Tissue was placed in 90% ethanol and retained for future genetic analysis.

2.3 Detection of A. astaci in crayfish specimens

DNA was extracted from soft abdominal cuticle of moribund or recently dead white-clawed crayfish specimens using the QIAamp DNA mini extraction kit and an automated QIAGEN Qiacube as per manufacturer's instructions. DNA extracts were screened for the presence of *A. astaci* using a Taqman real-time qPCR assay targeting a 59bp of the Internal Transcribed Spacer 1 (ITS) of the nuclear ribosomal gene cluster on a 7500 Real-Time PCR System (Applied Biosystems). In brief, 12.5 µl of TaqMan Universal Mastermix (Applied Biosystems) was used to make up a total volume of 25 µl containing 500nM of the forward (AphAstITS-39F, 5'-AAGGCTTGTGCTGGGATGTT-3') and reverse (AphAstITS-97R, 5'-CTTCTTGCGAAACCTTCTGCTA-3') primers, 200nM of the FAM-labelled MGB probe (AphAstITS-60T, 5'-TTCGGGACGACCC-3'), 1.5 µl molecular biology grade H_2O and 5 µl of DNA template.

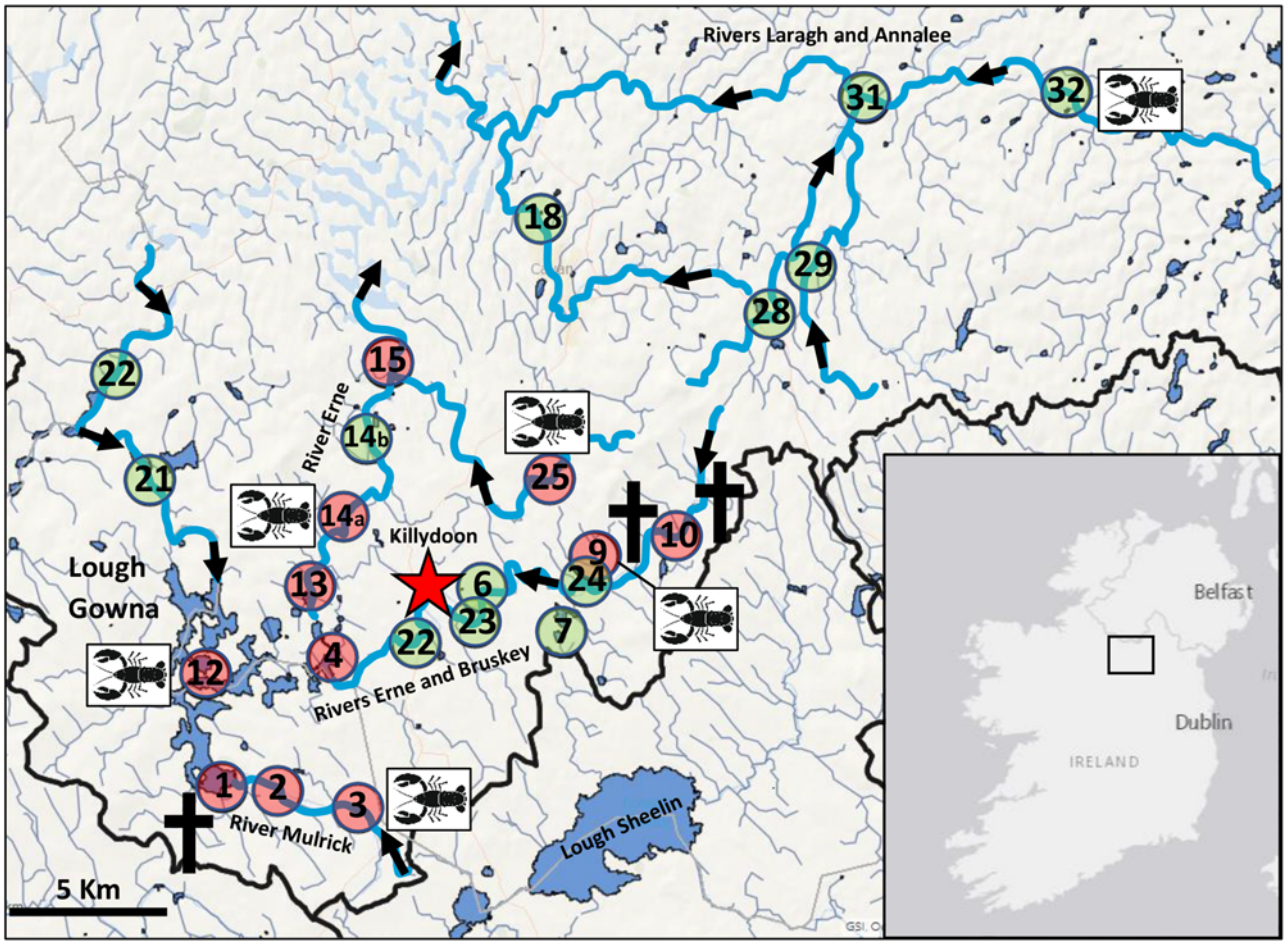

Fig. 1. Location of the 24 sites surveyed in 2016 (codes as shown in Tab. 1), and occurrence of live crayfish (crayfish icons), dead crayfish (crosses), sites positive and negative to crayfish plague's eDNA (red and green circles, respectively). Main rivers surveyed are shown as blue thick lines, direction of water flow is shown by black arrows and main catchment boundaries are shown by black lines. The red star indicates Killydoon location (epicentre of the original outbreak in 2015).

Lyophilised tissue infected with *A. astaci* (supplied by CEFAS) was utilised as the positive control material, while negative controls consisted of extraction reagents in the absence of template DNA. To ensure the quality of the tested samples and rule out any inhibition, an Internal Positive Control (IPC) (Applied Biosystems) with pre-designed primers and Taqman probe was used and an in house designed assay targeting a conserved region of the crustacean 18S ribosomal RNA gene to monitor sample extraction efficiency (Extraction Process Control, EPC) were run in parallel.

The specimen samples collected from the original outbreak site at Killydoon and from the subsequent survey further upstream were tested by conventional PCR according to the method described by Oidtmann *et al.* (2006), with some modifications. Briefly, a single round PCR was carried out with Primers 42 and 640: PCR amplifications were carried out in a 50 µl reaction volume containing AmpliTaq Gold PCR Mastermix and Primers 42 and 640 at a final concentration of 0.5 µM each. The mixture was denatured at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 58 °C, 1 min at 72 °C, and a final extension step of 7 min at 72 °C on a Biorad PTC-200 Thermocycler. The PCR products were separated by gel electrophoresis using 2% agarose gel and visualised on UV Transilluminator. Positive control material used was WCC pleopod tissue infected with *A. astaci* supplied by CEFAS UK (OIE Reference Laboratory for Crayfish plague). PCR products generated from conventional PCR using BO-42 and BO-640 primers were sent for commercial Sanger sequencing to Sequiserve GmbH, Germany. The resulting consensus sequence was submitted to GenBank (accession number ON062420-ON062421) and compared with published sequence alignments for *A. astaci* using BLAST to confirm identification as *A. astaci*.

2.4 Detection of A. astaci in the environment (eDNA)

Following an initial pilot eDNA sampling trial, a water collection and filtration protocol was developed and implemented to avoid cross-sample contamination of eDNA samples. Because of the presence of considerable amounts of suspended solids in the surveyed areas, the volume of water filtered per replicate was adjusted to 100 mL per replicate. Since the limit of detection of the assay is down to less than one *A. astaci* genome per reaction (Vrålstad *et al.*, 2009), this

Table 1. Details of locations and dates of site visits during the 2016 survey.

Site code	Lake/River name	Lat, Long	Date (visit 1)	Date (visit 2)
1	Lough Gowna	53.81911, -7.55319	24/08/2016	21/09/2016
2	River Mulrick	53.81803, -7.5214		
3	River Mulrick	53.81237, -7.47556		
4	Lough Gowna	53.86287, -7.4895		
6	River Erne	53.88656, -7.40474		
7	Kill Lough	53.87133, -7.35942		
9	River Erne	53.89718, -7.34049		
10	River Erne	53.90395, -7.29444		
12	Lough Gowna	53.85715, -7.56243	31/08/2016	28/09/2016
13	River Erne	53.88678, -7.50314		
14a	River Erne	53.91019, -7.48393		
14b	River Erne	53.9363, -7.47031		
15	River Erne	53.96169, -7.45825		
18	River Cavan	54.00995, -7.37098		
20	River Cullies	53.95808, -7.61348		
21	River Drumnawall	53.92251, -7.59451		
22	River Erne	53.86779, -7.44263	07/09/2016	13/09/2016
23	River Erne	53.8746, -7.40895		
24	River Erne	53.88867, -7.34566		
25	River Ballinagh	53.92282, -7.36651		
28	River Stradone	53.97809, -7.24046		
29	River Laragh	53.99389, -7.2169		
31	River Annalee	54.05057, -7.18636		
32	River Annalee	54.05228, -7.07193		

amount of water was deemed appropriate to capture and detect spores in the water column if present. At each site, 3 * 100 mL water samples were collected from the surface using sterile DNA-free polyethylene bottles, as well as an additional 100 mL sample collected at depth (*e.g.* as close to the bottom substrate as possible) and filtered through sterile Cellulose Nitrate membranes (0.45 µm pore size, 47 mm diameter, Thermo Scientific™ Nalgene™) using a portable peristaltic pump (Geopump™ Series II, Geotech). Water samples were filtered on site immediately or after storage in the dark in a cooler box within 12 h from collection. Upon filtration, filter membranes were transferred into pre-labelled sterile 15 ml tubes or zip-lock bags and kept on ice until final storage at –20 °C or –80 °C in GMIT laboratories. Daily field negative controls consisted of 100 mL distilled water stored and filtered as per above protocol and repeated at every visit (*i.e.* one field negative per day of collection). Prior to choosing the laboratory screening protocol (from extraction to data), preliminary validation steps were carried out to assess potential inhibition effects and sensitivity of the eDNA assay. Ultimately, the extraction protocol chosen for isolating eDNA from membranes was the PowerWater kit (MO BIO; now DNeasy Power Water kit, QIAGEN). Extractions were carried out from half membrane and the final elution step was done with 50µl of elution buffer (PW6), while the other half of the membranes were retained as a backup sample. Prior to DNA extractions, surfaces and instruments were cleaned with a 5% sodium hypochlorite solution and 70% ethanol. All extractions were performed using sterile DNAse-free filter tips under a laminar flow hood and in a separate room from subsequent downstream applications (*e.g.* PCR and qPCR). The same qPCR assay described above (Vrålstad *et al.*, 2009) was used for the detection of the crayfish plague (*A. astaci*) in eDNA samples. In this case, qPCR conditions included 1X qPCR master mix (Promega), 500 nM of each primer and 250 nM of probe (PrimeTime™, Integrated DNA Technologies, Inc.) and two microliters of template eDNA. All reactions were carried out in the presence of laboratory negative controls (no template control). For each qPCR run, technical No Template Controls were included and standard curves were generated using serial dilutions of a synthetically-derived 493bp portion of the ITS gene (gBlocks™, Integrated DNA Technologies, Inc.) encompassing the target region of the assay. All reactions and qPCR run parameter analysis were performed using a Rotor-Gene™ 6000 Real-Time qPCR and associated software (Corbett Life Science). A sample was deemed positive if any of the field replicates was positive for at least one of the two laboratory technical replicates and showed a higher value than the Limit Of Detection (LOD). The LOD of the crayfish plague assay was determined by replicating (n = 9) serial dilutions of

the synthetic gBlock target, ranging from 0.02 pg/µl to 0.000128 fg/µl. Theoretical LOD estimates were then determined following a strict 95% positive rule as well as the approach of Klymus *et al.* (2020) using R Studio (v. 1.0.143). Estimated DNA concentration (ng/µl) were converted into copies of target DNA/µl using the following formula: (est. ng of DNA * 6.022 * 10^{23})/(target length bp * 1 * 10^{9} * 650).

3 Results

3.1 Conventional (direct) surveys

Following the first outbreak in 2015, approximately 600 dead crayfish were found in a relatively small stretch of the River Bruskey, Co. Cavan. Sequencing of PCR products from two dead individuals confirmed that the mortality event was due to infection by *Aphanomyces astaci*, the causative agent of the crayfish plague. In subsequent surveys carried out in 2015, the recovery of dead crayfish revealed that the spread of the plague progressed rapidly several kilometers upstream, but appeared not to have spread downstream of the outbreak's epicentre, possibly due to the buffering/dilution action of Lough Gowna. During the 2017 follow-up survey, live and apparently healthy WCC were still present in the affected areas but abundances appeared to be low compared to original numbers (data not shown). The expected spread of the plague in the studied areas over the period surveyed is shown in Figure 1.

3.2 Environmental DNA (indirect) surveys

The areas and sites surveyed in conjunction with eDNA analysis comprised mostly shallow fast flowing streams, except for Lough Gowna (sites 1, 4 and 12) and Kill Lough (site 7). Overall, substrates included a mixture of boulders, cobbles, pebbles, gravel and sand, and macrophytes were present on average over 23.6% of surveyed areas. The presence of predators and crayfish prey remains (otter spraints) was observed in site 32. Environmental parameters showed a high range of variability between and within sites, with surface water temperatures of 11.9–20.7 °C, DO 4.2–15.1 mgO_2/L, % saturation 47.3–167.6, conductivity 131.9–584 µS/cm, pH 7.3–10.1, absorbance at 540 nm 0.003–0.033, absorbance at 750 nm 0.002–0.036.

No mass mortality was observed during the 2016 survey period, however, a number of dead WCC individuals were found at the first visit to sites 1 (n = 2 fresh), 9 (n = 1 fresh) and 10 (n = 1 partly decomposed/remains). These four individuals tested positive for *A. astaci* by means of real-time qPCR analysis. Using conventional capture methods (hand search, hand nets and trapping), live WCC were found in six out of 24 sites surveyed; these were sites 3, 9, 12, 14a, 25 and 32 (Tab. 2 and Fig. 1). Overall, numbers of captured white-clawed crayfish ranged from one to 19 per site per visit and showed no apparent signs of infection (*e.g.* lively escaping behaviour and no lethargy). Most sites showed absence of crayfish or very low abundance (Tab. 2), except for site 32 (located in the River Annalee, which is in a separate sub-catchment to the Bruskey (Fig. 1)), which showed relatively high numbers of individuals. No other species of crayfish (*i.e.* NICS) were found during the survey period using conventional methods.

Following the conditions used in the present study, results of the performance of the eDNA assay at a range of target DNA concentrations is shown in Table 3. LOD was determined to be at 60 target DNA copies/µl (mean Cq = 35.74) (following a strict 95% rule) or 25 copies/µl (mean Cq = 40.26) (following the method of Klymus *et al.* 2020). From the 24 sites, a total of 168 water samples were collected (including 5 field negative controls) and screened for the presence of *A. astaci* eDNA. The pathogen was detected in 11 out of 24 sites, including five of the six sites where live crayfish were captured by conventional methods (Tab. 4).

4 Discussion

The present study confirms that *A. astaci* was the causative agent of the WCC mass mortality event in the Bruskey River in 2015. Although some mass mortality events have been previously reported in Ireland in the 1980s (Reynolds, 1988), this is the first official confirmation of the crayfish plague in Ireland at least in the past 30 years. This event led to a drastic decline of the local WCC population bringing it to levels close to local extinction, however the post-outbreak persistence of some individuals in the affected areas suggests some level of potential resistance to the pathogen, which was reported in other studies (Martín-Torrijos *et al.*, 2017). Several strains of *A. astaci* have been described to date, some of which appear to present variable levels of virulence and can be found in both native and non-native crayfish populations (Kozubíková *et al.*, 2011; Maguire *et al.*, 2016). Thus, further investigations are required to elucidate the actual strain that affected the Bruskey area, which may explain some level of resistance and survival by the local WCC population. *A. astaci* was detected in dead WCC as well as in water samples (eDNA) in five out of six sites (where live and apparently healthy WCC were found), indicating that the pathogen was still present in the system one year after the original outbreak in 2015. Nonetheless, numbers were still very low compared to the original population density, which was assumed to be in the hundreds based on the number of carcasses found in the Killydoon area in 2015. This indicates that the local population has undergone a severe demographic bottleneck, as it has been observed in other studies of European crayfish (Cammà *et al.*, 2010; Diéguez-Uribeondo *et al.*, 1997; Grandjean *et al.*, 2017; Kozubíková *et al.*, 2008).

The observed pattern of disease spread post-outbreak is similar to that reported in other studies, whereby mortalities are observed both down- and upstream of the epicentre, but also that local hydrographical features play an important role (*e.g.* Collas *et al.*, 2016). Immediately after the original outbreak in 2015, the spread of the pathogen (as shown by the presence of mortalities) was fast and facilitated by water current, despite measures being taken by NPWS to inform stakeholders and prevent further spread. The mode of spread was downstream but also upstream, probably facilitated by individual movement. Even though the drastic reduction in effective population size may have caused a significant genetic bottleneck, the local population could be restored either by enabling survivors to repopulate the area and/or by careful

Table 2. Detection of live crayfish during the survey period using conventional capture methods. Traps CPUE was calculated over one day (visit 1) or five days (visit 2).

		Number of indivduals found by hand and net search (5 minutes)		Traps CPUE (individuals per day)	
Site code	River/Lake name	Visit 1	Visit 2	Visit 1	Visit 2
1	Lough Gowna				
2	River Mulrick				
3	River Mulrick	2	1		0.4
4	Lough Gowna				
6	River Erne				
7	Kill Lough				
9	River Erne		1		
10	River Erne				
12	Lough Gowna				0.2
13	River Erne				
14a	River Erne	4	1		0.2
14b	River Erne				
15	River Erne				
18	River Cavan				
20	River Cullies				
21	River Drumnawall				
22	River Erne				
23	River Erne				
24	River Erne				
25	River Ballinagh			2	0.2
28	River Stradone				
29	River Laragh				
31	River Annalee				
32	River Annalee	9.5			1.4

restocking practices (Manenti *et al.*, 2021; Reynolds *et al.*, 2002, 2009; Souty-Grosset and Reynolds, 2009), although it may be prudent to continue monitoring for the presence of *A. astaci* before restocking efforts commence. Maintaining healthy populations of WCC in Ireland is not only important for the preservation of local ecosystems but also because Irish WCC populations have been suggested as potential source stocks for reintroduction in other European areas that are recurrently affected by pathogens and non-indigenous carrier species (Reynolds *et al.*, 2002).

During the 2016 survey, a healthy population of WCC was found in the River Annalee, which is a different sub-catchment of the Erne system compared to the Erne, suggesting that the pathogen was still contained within the Bruskey sub-catchment. However, the survey conducted in 2016 not only confirmed the presence of the pathogen around the original outbreak epicentre one year later, but also showed that it had spread downstream into and below Lough Gowna. The persistence of the pathogen and its spread in larger water bodies increases the chances of further spread of viable *A. astaci* spores to additional water courses through contamination of wet gear and equipment used by recreational water users. Following the Bruskey outbreak, several further WCC crayfish plague events have occurred across Ireland (https://www.biodiversityireland.ie/projects/invasive-species/crayfish-plague/). Ongoing monitoring and investigation are taking place to shed light on whether the Bruskey outbreak acted as a source or is related to other events that have been occurring around the country.

While it is still possible that a small number of NICS were present in the system, the present study did not find any evidence of NICS in the surveyed area (using trapping and hand searching), suggesting that the introduction of the pathogen may have occurred by introduction of contaminated wet gear bearing viable spores or hyphae), rather than by the presence of infected carrier NICS, which would otherwise be

Table 3. qPCR results (Cq values) of theoretical Limit Of Detection (LOD) estimation.

	Concentration of target DNA							
Est. Conc	**0.02 pg/µl**	**4 fg/µl**	**0.8 fg/µl**	**0.16 fg/µl**	**0.032 fg/µl**	**0.0064 fg/µl**	**0.00128 fg/µl**	**NTC**
Est. copies of DNA	**314055**	**62811**	**12562**	**2512**	**502**	**100**	**20**	**0**
Replicate 1	26.64	30.84	33.79	35.25	38.87	41.34	neg	neg
Replicate 2	26.73	29.99	34.13	36.21	40.39	41.44	neg	neg
Replicate 3	26.6	30.62	33.45	35.53	37.91	neg	neg	neg
Replicate 4	26.78	30.35	33.6	35.8	38.74	38.71	neg	neg
Replicate 5	26.93	30.2	33.88	36.99	41.04	38.75	neg	neg
Replicate 6	26.79	30.11	33.77	34.8	41.4	neg	neg	neg
Replicate 7	27.03	30.17	33.51	35.99	39.96	neg	neg	neg
Replicate 8	27.16	30.09	30.8	35.49	neg	43.45	neg	neg
Replicate 9	26.85	30.57	29.51	35.58	40.13	neg	neg	neg
Mean	26.83	30.33	32.94	35.74	39.81	40.74	neg	neg
Standard deviation	0.18	0.29	1.62	0.62	1.20	2.02	neg	neg

Table 4. Cq values for positive A. astaci eDNA detection, below the "strict" LOD (red) and below the "relaxed" LOD (orange).

	Rep 1	Rep 2	Rep 3	Depth
Site 1			36.64	
Site 2		36.52	36.08	
Site 3	36.31			36.57
Site 4			36.27	
Site 6				
Site 7				
Site 9		35.23		35.73
Site 10	38.19	37.54		
Site 12	36.44			
Site 13		36.38	36.54	
Site 14a	35.32			
Site 14b				
Site 15				36.54
Site 18				
Site 20				
Site 21				
Site 22				
Site 23				
Site 24				
Site 25	35.22			
Site 28				
Site 29				
Site 31				
Site 32				

difficult to eradicate as is the case in Britain (James *et al.*, 2017). *A. astaci* is characterised by genetic intraspecific variation, whereby specific genetic variants seem to be associated to specific intermediate hosts/carrier species (Svoboda *et al.*, 2017). Thus, to further elucidate the origin of the pathogen current work is being undertaken to investigate the pathogen's genetic variants in the various Irish plague outbreak events. The eDNA approach was effective at detecting water borne traces of the pathogen, however for the positive sites not all replicates were positive, which could

be explained by a low concentration of the pathogen in the water column. Larger volumes of water could not be processed at the time of the survey due to high amounts of suspended solids in the water column, however, in case of turbid waters, it is recommended to aim for larger volumes of water in future surveys (*e.g.* increasing the number of replicates and/or increasing the pore size of the filter membranes). Additionally, the eDNA approach has shown to be effective at detecting host crayfish species (including NICS) and also to be superior to conventional trap-based assessment approaches in mainland Europe and Britain, respectively (Atkinson *et al.*, 2019; Robinson *et al.*, 2018; Wittwer *et al.*, 2018). This is the first field study to apply eDNA techniques aiming to identify the pathogen *A. astaci* from water samples in the Republic of Ireland. Considering these data, the implementation of preliminary eDNA monitoring using water samples from a broad range of sites could identify both *A. astaci* and potential NICS hosts in parallel, providing a valuable tool to pinpoint sites of interest for more direct and comprehensive sampling methods.

In addition to other ongoing activities aimed at reducing risks associated to biosecurity (*e.g.* the "Check, Clean, Dry" campaign, https://invasives.ie/check-clean-dry-resources/), a number of measures are hereby recommended for future monitoring and management of this threat to Irish WCC populations, including: (i) increase stakeholder awareness on native protected fauna as well as risks associated to anthropogenic activities; (ii) promote biosecurity practices for commercial and recreational water users; and (iii) increase monitoring and continue documenting the spread, including the establishment of reference material/data for future genetic screening.

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References

Atkinson S, Carlsson JEL., Ball B, Kelly-Quinn M, Carlsson J. 2019. Field application of an eDNA assay for the threatened white-clawed crayfish *Austropotamobius pallipes*. *Freshw Sci* 38: 503–509.

Ballesteros I, Martín MP, Cerenius L, Söderhäll K, Tellería MT, Diéguez-Uribeondo J. 2007. Lack of specificity of the molecular diagnostic method for identification of *Aphanomyces astaci*. *Bull Fr la Pech la Prot des Milieux Aquat*. 385: 17–24.

Becking T., Kiselev A., Rossi V., Street-Jones D., Grandjean F., Gaulin E. 2021. Pathogenicity of animal and plant parasitic *Aphanomyces* spp and their economic impact on aquaculture and agriculture. *Fungal Biol Rev.* https://doi.org/10.1016/J.FBR.2021.08.001

Bouallegui Y. 2021. A comprehensive review on crustaceans' immune system with a focus on freshwater crayfish in relation to crayfish plague disease. *Front Immunol* 0: 1753.

Cammà C., Ferri N., Zezza D., *et al.* 2010. Confirmation of crayfish plague in Italy: detection of Aphanomyces astaci in white clawed crayfish . *Dis Aquat Organ* 89: 265–268.

Collas M., Becking T., Delpy M., *et al.* 2016. Monitoring of white-clawed crayfish (*Austropotamobius pallipes*) population during a crayfish plague outbreak followed by rescue. *Knowl Manag Aquat Ecosyst* 417: 1.

Diéguez-Uribeondo J., Temiño C., Múzquiz J.L. 1997. The crayfish plague fungus (*Aphanomyces astaci*) in Spain. BFPP - *Bull. Fr. la Pech. la Prot. des Milieux Aquat*. 70: 753–763.

Filipová L., Petrusek A., Matasová K., Delaunay C., Grandjean F. 2013. Prevalence of the crayfish plague pathogen aphanomyces astaci in populations of the signal crayfish *Pacifastacus leniusculus* in France: evaluating the threat to native crayfish. *PLoS One* 8: e70157.

Gouin N., Grandjean F., Pain S., Souty-Grosset C., Reynolds J. 2003. Origin and colonization history of the white-clawed crayfish, *Austropotamobius pallipes*, in Ireland. *Heredity* (Edinb). 91: 70–77.

Grandjean F., Gilbert C., Razafimafondy F., *et al.* 2019. A new bunya-like virus associated with mass mortality of white-clawed crayfish in the wild. *Virology* 533: 115–124.

Grandjean F., Roques J., Delaunay C., Petrusek A., Becking T., Collas M. 2017. Status of *Pacifastacus leniusculus* and its role in recent crayfish plague outbreaks in France: improving distribution and crayfish plague infection patterns. *Aquat Invasions* 12: 541–549.

Hochwimmer G., Tober R., Bibars-Reiter R., Licek E., Steinborn R. 2009. Identification of two GH18 chitinase family genes and their use as targets for detection of the crayfish-plague oomycete *Aphanomyces astaci*. *BMC Microbiol* 91: 1–17.

Holdich D.M., Rogers W.D. 1997. The white-clawed crayfish, *Austropotamobius pallipes*, in Great Britain and Ireland with particular reference to its conservation in Great Britain. *Bull Français la Pêche la Piscic* 70: 597–616.

James J., Nutbeam-Tuffs S., Cable J., *et al.* 2017. The prevalence of *Aphanomyces astaci* in invasive signal crayfish from the UK and implications for native crayfish conservation. *Parasitology* 144: 411–418.

Kaldre K., Paaver T., Hurt M., Grandjean F. 2017. First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia. *Biol Invasions* 19: 2771–2776.

Klymus K.E., Merkes C.M., Allison M.J., *et al.* 2020. Reporting the limits of detection and quantification for environmental DNA assays. *Environ DNA* 2: 271–282.

Kouba A., Petrusek A., Kozák P. 2014. Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl Manag Aquat Ecosyst* 413: 05.

Kozubíková E., Petrusek A., Ďuriš Z., Martín M.P., Diéguez-Uribeondo J., Oidtmann B. 2008. The old menace is back: Recent crayfish plague outbreaks in the Czech Republic. *Aquaculture* 274: 208–217.

Kozubíková E., Viljamaa-Dirks S., Heinikainen S., Petrusek A. 2011. Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *J Invertebr Pathol* 108: 214–216.

Lowe S., Browne M., Boudjelas S., De Poorter M. 2004. 100 of the world's worst invasive alien species a selection from the global invasive species database. The invasive species specialist group (ISSG) of the world conservation union (IUCN), Auckland.

Maguire I., Jelić M., Klobučar G., Delpy M., Delaunay C., Grandjean F. 2016. Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia. *Dis Aquat Organ* 118: 45–53.

Manenti R., Barzaghi B., Nessi A., Cioccarelli S., Villa M., Ficetola G.F. 2021. Not only environmental conditions but also human awareness matters: a successful post-crayfish plague reintroduction of the white-clawed crayfish (*Austropotamobius pallipes*) in Northern Italy. *Front Ecol Evol* 0, 64.

Martín-Torrijos L., Campos Llach M., Pou-Rovira Q., Diéguez-Uribeondo J. 2017. Resistance to the crayfish plague, *Aphanomyces astaci* (*Oomycota*) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS One* 12: 1–13.

Matthews M., Reynolds J.D. 1992. Ecological impact of crayfish plague in Ireland. *Hydrobiology* 234: 1–6.

Matthews M., Reynolds J.D. 1990. Laboratory investigations of the pathogenicity of *Aphanomyces astaci* for Irish freshwater crayfish. *Hydrobiology* 203: 121–126.

Oidtmann B., Bausewein S., Hölzle L., Hoffmann R., Wittenbrink M. 2002. Identification of the crayfish plague fungus *Aphanomyces astaci* by polymerase chain reaction and restriction enzyme analysis. *Vet Microbiol* 85: 183–194.

Oidtmann B., Geiger S., Steinbauer P., Culas A., Hoffmann R.W. 2006. Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Dis Aquat Organ* 72: 53–64.

Panteleit J., Keller N.S., Kokko H., *et al.* 2017. Investigation of ornamental crayfish reveals new carrier species of the crayfish plague pathogen (*Aphanomyces astaci*). *Aquat Invasions* 77–83.

Reynolds J.D. 1998. Conservation management of the white-clawed crayfish, *Austropotamobius pallipes*. Part 1. *Irish Wildlife Manuals*, No. 1.

Reynolds J.D. 1988. Crayfish extinctions and crayfish plague in central Ireland. *Biol Conserv* 45: 279–285.

Reynolds J.D., Demers A., Marnell F. 2002. Managing an abundant crayfish resource for conservation – *A. pallipes* in Ireland. *Bull Français la Pêche la Piscic*. 367: 823–832.

Reynolds J.D., Souty-Grosset C., Gouin N., Devaney S., Grandjean F. 2009. Experimental restocking of native crayfish in White lake, Co. Westmeath, Ireland, in: Brickland J., Holdich D.M. and Imhoff E.M. (Eds) (2009). Crayfish Conservation in the British Isles. Proceedings of a *Conference Held on 25th March 2009 in Leeds, U.K.*

Robinson C.V., Uren Webster T.M., Cable J., James J., Consuegra S. 2018. Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biol Conserv* 222: 241–252.

Souty-Grosset C., Holdich D.M., Noël P.Y., Reynolds J., Haffner P. 2006. Atlas of crayfish in Europe. Muséum National d'Histoire Naturelle, Paris.

Souty-Grosset C., Reynolds J.D. 2009. Current ideas on methodological approaches in European crayfish conservation and restocking procedures. *Knowl Manag Aquat Ecosyst* 01. https://doi.org/10.1051/KMAE/2009021

Strand D.A., Holst-Jensen A., Viljugrein H., *et al.* 2011. Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Dis Aquat Organ* 95: 9–17.

Strand D.A., Jussila J., Viljamaa-Dirks S., *et al.* 2012. Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Vet Microbiol* 160: 99–107.

Svoboda J., Mrugała, A., Kozubıkova-Balcarova E., Petrusek A. 2017. Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J Fish Dis* https://doi.org/10.1111/jfd.12472

Vrålstad T., Knutsen A.K., Tengs T., Holst-Jensen A. 2009. A quantitative TaqMan MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague Aphanomyces astaci. *Vet Microbiol* 137: 146–155.

Wittwer C., Stoll S., Strand D., Vrålstad T., Nowak C., Thines M. 2018. eDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. *Hydrobiologia* 807: 87–97.

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