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Short Communication

eDNA-based detection as an early warning tool for detecting established and emerging invasive amphipods

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Abstract

Introductions of invasive non-native species often have severe effects on environments and can lead to the collapse of local populations. Freshwater systems are highly biodiverse habitats that are particularly sensitive to the introduction of non-native species, which is recognized as one of the leading causes of animal extinctions worldwide. Effective management firstly depends on the early detection of incipient invasions and subsequent rapid response. Here, we focus on the cryptic and difficult-to-detect amphipod, *Crangonyx pseudogracilis,* which is already established in several countries in Europe, and *C. floridanus*, which was recently found in the UK and Ireland. Their exact distribution is relatively unknown, and their morphological similarities to each other makes them taxonomically difficult to distinguish. To obtain better insights on the actual distribution of both species, we developed and validated species-specific assays for the detection of environmental DNA traces from both *C. floridanus* and *C. pseudogracilis*. Next, we analysed a large number of eDNA samples collected from several freshwater systems in Ireland where the presence of both alien species is known, and from unknown sites in Belgium, Netherlands, France, and Norway (countries where only *C. pseudogracilis* has previously been recorded). Using qPCR analyses, we successfully detected eDNA from both *C. pseudogracilis* and *C. floridanus* in the known locations in Ireland. Sites investigated in Belgium, Netherlands, France, and Norway were negative for the presence of both species. The qPCR based detection of freshwater amphipods from aquatic eDNA is a cost-effective and sensitive method to monitor establishment and spread of invasive species as well as species of conservation priority. Based on our results, we recommend that eDNA surveys can be used as a powerful tool for stakeholders, including ecologists, especially in at-risk areas to detect potential early invasion of these species.

Key words: invasive species, environmental DNA, qPCR, *Crangonyx*, freshwater habitats

Introduction

The introduction of invasive non-native species has significant negative impacts on indigenous communities and ecosystems worldwide (Johnson et al. 2009; Gallardo et al. 2016; Baudry et al. 2021). In freshwater ecosystems,

which generally harbour large numbers of distinct animal and plant species, these effects are often exacerbated and have been shown to threaten ecosystem services (Moorhouse and Macdonald 2015; Kiruba-Sankar et al. 2018). Newly invasive crustaceans, for example, have previously shown to decrease local biodiversity due to predation, competition or spread of disease (Bailey et al. 2006; Williams et al. 2006; MacDonald et al. 2007; Strecker and Arnott 2008; Pintor and Sih 2009; Bacela-Spychalska et al. 2012, 2012; Iacarella et al. 2015; Kiruba-Sankar et al. 2018). These species often show the capacity to disperse over long distances through ballast water, aquaculture, pet trade, or recreational boats (Panov et al. 2004; Holdich and Pöckl 2007; Kelly et al. 2013; Bailey 2015). Crustaceans have been shown to be the main stowaways in cargo ballast water through larval transport (Panov et al. 2004; DiBacco et al. 2012), enhancing the introduction of many species into non-native areas (Holdich and Pöckl 2007; Gollasch et al. 2015). Consequently, alien crustacean species are now being recorded in all European countries, with new species being regularly discovered (Martínez and Adarraga 2008; Bódis et al. 2012; Lipták 2013; Novitsky and Son 2016). Knowledge of these species' presence is critical to ensure appropriate management decisions and efficient conservation of native ecosystems and environments (Hulme 2009; Rodríguez-Rey et al. 2019; Sepulveda et al. 2020). Compared to other taxa, relatively little attention has been paid to the introduction of amphipods into inland waters and many invasive amphipods remain undetected in European freshwater systems (Holdich and Pöckl 2007; Cuthbert et al. 2020). This can be attributed to their cryptic behaviour and difficulties for appropriate identification and monitoring. As a result, the invasive amphipod *Crangonyx floridanus* (Bousfield, 1963) was recorded for the first time in the United Kingdom in 2017 and in Ireland in 2021 (Mauvisseau et al. 2019b; Baars et al. 2021). Although the invasive *C. floridanus* was only detected in the last five years, it likely remained unnoticed for a long period due to having a morphology similar to the previously established invasive *C. pseudogracilis* (Bousfield, 1958). The first was likely never detected due to misidentification, and silently spread in these two countries before being detected. Indeed, morphological identification can be challenging in cases of highly similar species, and this is especially the case for *C. floridanus* and *C. pseudogracilis* (Mauvisseau et al. 2019b). The distinction is challenging as only mature *C. pseudogracilis* males can be distinguished from *C. floridanus* specimens through the presence of ventral spines on the outer ramus of the second uropod (Mauvisseau et al. 2019b). Hence, accurate identification of any of these two species in the field is extremely challenging, if not impossible, and requires a high level of taxonomic expertise (Nagakubo et al. 2011; Cannizzaro et al. 2020).

Crangonyx floridanus has not only spread to the United Kingdom and Ireland but across the world. The first time it was recorded outside of its

native range in North America was in 1989, when the species was detected in Japan (Morino et al. 2004). Since then, this invasive amphipod has undergone rapid expansion in Japan and has, over a period of 30 years, been recorded in nearly the entire archipelago, although it is suggested that the species spread faster than first anticipated (Kanada et al. 2007; Nagakubo et al. 2011). Its high population growth rate is attributed to its efficient breeding and ability to survive in a wide range of water temperatures and water flow rates (Tojo et al. 2010; Nagakubo et al. 2011). Reports of cases where eggs of the next-cycle offspring were oviposited within one day of previous-cycle offspring hatching and separating from the female parent suggest a very high reproductive fecundity (Tojo et al. 2010). This is further coupled with its ability to survive in temperatures as low as 4 °C and up to 30 °C (Tojo et al. 2010). A high grazing rate and varied diet, combined with its rapid population growth and dispersal, makes *C. floridanus* a significant threat to native species occupying similar niches (Nagakubo et al. 2011; Baars et al. 2021). Due to its high potential to disrupt indigenous ecosystems, an accurate distribution of *C. floridanus* in new ecosystems is imperative to mitigate negative impacts on biodiversity (Baars et al. 2021).

However, providing such information comes with challenges. Amphipods can show patchy distributions, and high variations in abundance can lead to difficulties in detection. Although *C. pseudogracilis* was detected in the United Kingdom as early as 1936 (Crawford 1937; Zhang 1997) similar morphology of *C. floridanus* and *C. pseudogracilis* makes it hard to rule out that *C. floridanus* had not been introduced to the British Isles prior to 2017. Additionally, visual identification relies on the physical capture of specimens through kick sampling, netting, or trapping (Mächler et al. 2014). This results in targeted and non-targeted co-occurring species likely to be injured by such ecologically invasive survey methods (Deiner et al. 2013; Mächler et al. 2014). To bypass this, molecular based identification tools such as the use of environmental DNA (eDNA) monitoring, can be used to both detect and reliably identify these amphipod species.

Environmental DNA (eDNA) monitoring relies on the detection of DNA traces left by organisms in their environment (Lodge et al. 2012; Taberlet et al. 2012; Thomsen and Willerslev 2015). DNA can be sampled and extracted from various media such as water (freshwater or marine; Mächler et al. 2014), ice (Willerslev et al. 2007), sediments (Turner et al. 2015), air (Lynggaard et al. 2022), spider-webs (Xu et al. 2015) or even sponges (Cai et al. 2022). Detection, and in some cases quantification of DNA, from targeted species can be obtained through PCR (Mächler et al. 2014), qPCR (Minamoto et al. 2019) or ddPCR (Doi et al. 2015) analysis, while community composition is obtained through high-throughput sequencing (Sogin et al. 2006). Species-specific approaches using qPCR and ddPCR have been more commonly applied due to its accuracy, efficiency and cost benefits compared to traditional monitoring, especially in freshwater systems (Taberlet et al. 2012; Mächler

et al. 2014; Rees et al. 2014; Handley 2015; Thomsen and Willerslev 2015; Harper et al. 2019; Mauvisseau et al. 2019a; Brys et al. 2021; Nordstrom et al. 2022; Rourke et al. 2022). Here, the aims of our study were to (i) develop and validate species-specific assays of two freshwater crangonyctid, *C. floridanus* and *C. pseudogracilis,* that are difficult to identify based on morphological features alone, (ii) to test whether these species can appropriately be detected via eDNA traces in water samples, and (iii) use both primer/probe assays for the detection of both species in a large number of eDNA samples collected in several locations spanning four European countries with known and unknown presence of the two species.

Materials and methods

Primers and probe design

Species-specific primers and probes targeting the COI marker from *C. floridanus* and *C. pseudogracilis* were designed following the methods outlined in Brys et al. (2021) and Mauvisseau et al. (2020) using the Geneious Pro R10 software (https://www.geneious.com; Kearse et al. 2012). Primers and probes targeting *C. floridanus* (Forward: 5' - CCTCGTTAT GTCTTCTGCTT - 3', Reverse: 5' - AGCACCTCTATGAGCAGCTGT - 3', FAM labelled Probe: 5' - ACTGTCTACCCGCCTTTAGCA - 3') and *C. pseudogracilis* (Forward: 5' - GAGCTATCAATTTTCTATCCAC - 3', Reverse: 5' - AGAGACAGAAGGAGAAGAATTG - 3', HEX labelled Probe: 5' - ACCTTCTTATAGACCAAGTTC - 3') were designed using COI sequences deposited in GenBank (Supplementary material Table S1), and visual alignment of the primers/probes and COI sequences can be found in Figures S1, S2. As in Mauvisseau et al. (2020) and Brys et al. (2021), consensus sequences from targeted and co-occurring species were used to increase the primers and probe specificity. In brief, these consensus sequences were aligned using the Geneious software mentioned above with the "multiple alignment" function, and primers/probes were designed on COI fragments variable across species (highlighted in Table S1). This was done using the "primers" design function of the software. Additionally, specificity was further assessed by visual alignment with sequences used while designing primers (Table S1), and through *in-silico* testing using the NCBI primer-blast function (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Finally, *in-vitro* assessment was conducted using DNA extracted from *C. floridanus, C. pseudogracilis* and the co-occurring species, *Gammarus tigrinus*, *G. duebeni*, *G. lacustris*, and *G. pulex*, to confirm that the designed primers and probes were species-specific. DNA was extracted from *C. floridanus* (n = 1), *C. pseudogracilis* (n = 5), *Gammarus tigrinus* (n = 3), *G. duebeni* (n = 3), *G. lacustris* (n = 6), and *G. pulex* (n = 3) specimens collected in Ireland, using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's instructions (it should be noted that COI sequences

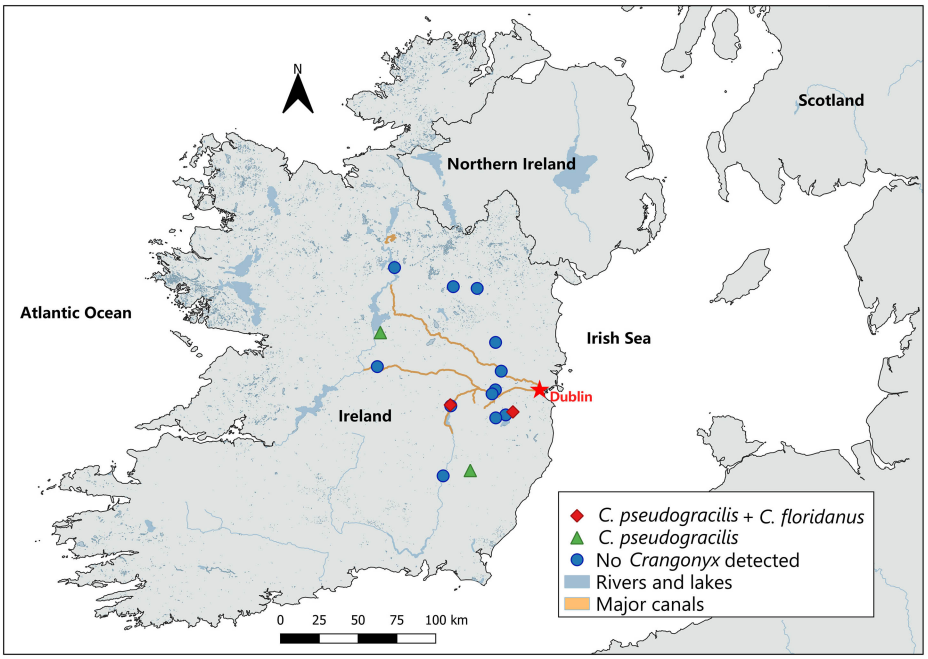

Figure 1. Map showing the locations sampled across Ireland. Diamonds in red show where both *C. floridanus* and *C. pseudogracilis* were detected, triangles in green show where only *C. pseudogracilis* was detected, and circles in blue show where none of *Crangonyx* species investigated in our study were detected. For details see Table S2.

of all *C. floridanus* collected in Europe (Table S1) show no sequence divergence on the targeted COI region). DNA extracts were used for generation of DNA reference barcodes to confirm species identification (see Appendix 1). Finally, we tested the possibility of our designed assays to cross-amplify potentially co-occurring or closely related species listed above using a machine learning tool developed in Kronenberger et al. (2022).

eDNA samples

A total of 84 eDNA samples from 49 sites distributed across freshwater systems such as rivers, lakes, and canals in Belgium, Netherlands, France, Ireland, and Norway were sampled (Appendix 1, Table S2). All sampling locations are shown in Figure S3, and a focus of the locations sampled in Ireland in Figure 1. We collected 20 samples ranging from 300 mL to 2000 mL spread across 18 locations in Belgium and 2 locations in the Netherlands between Spring 2018 and Summer 2021. A total of 10 samples ranging from 20 mL to 300 mL were sampled across 5 locations in France between December 2020 and January 2021. 33 samples ranging from 180 mL to 250 mL were sampled across 17 locations in Ireland in June 2021. Finally, a total of 21 samples ranging from 105 mL to 200 mL spread across 7 locations in Norway were collected in June 2021. All samples were collected from surface water to avoid disturbing sediments and potential turbidity of deeper layers in the water column. This was done to decrease potential inhibition effects. Further details regarding the sampling protocol, locations, replication, time of sampling and extraction protocols are outlined in the Appendix 1

section "Extraction protocols and other information related to the eDNA samples from each country", and Table S2 in Supplementary material.

PCR and qPCR amplification

To assess the specificity of primers and probes, qPCR amplification of DNA extracted from targeted and non-targeted species (n = 13) was performed. Additionally, qPCR amplification of eDNA samples (n = 84) was performed for each filter, running the single extracts from each filter in a quadruplicate for the qPCR step. To identify potential inhibition effects, eDNA samples from Ireland (where recent sightings of *C. floridanus* and *C. pseudogracilis* have been recorded) were additionally analysed with a 1:2 dilution of DNA template. Each plate also included two positive controls for *C. floridanus*, two positive controls for *C. pseudogracilis*, and four negative controls to monitor potential contamination and assess the efficiency of the amplifications (Dubreuil et al. 2022). Following primers/probes validation, the set of primers/probe was tested at different concentrations (0.27 and 0.3 µM final concentration for each primer and probe) and different annealing temperatures (from 55 to 60 °C) as in Baudry et al. (2021). The qPCR analyses were multiplexed, i.e., DNA from *C. floridanus* and *C. pseudogracilis* were amplified in a single reaction using species-specific primers, and species-specific probes with different fluorescence allowed the detection of each single species. All multiplex qPCR amplifications were completed on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, California, United States). The qPCRs were conducted in a 12.0 µL final volume containing 2.0 µL ddH_2O, 6.0 µL Bio-Rad SsoAdvanced Universal Probes Supermix, 0.3 µL of each forward and reverse primer (10 µM) and probes (10 µM), and 2.0 µL template DNA, using the following program: Initial denaturation at 98 °C for 2.5 min, followed by 45 cycles of denaturation at 98 °C for 10 sec, annealing at 55 °C for 30 sec and extension at 65 °C for 5 sec. All positive eDNA replicates were sent to Macrogen Europe B.V. for sequencing to further validate the primers/probe specificity and detection results.

qPCR sensitivity and analysis

To determine the limits of detection (LOD) and limits of quantification (LOQ), we conducted a serial dilution of DNA extracted from tissue samples of *C. floridanus* (starting concentration of 9.24 ng μl^{-1}, Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, California, United State) and *C. pseudogracilis* (starting concentration of 11.0 ng μl^{-1}, Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, California, United State) as in Baudry et al. (2021). This was the starting point for a twelve-step series of 5-fold dilutions (e.g., 1, 1/5, 1/25, etc.) using 10 qPCR replicates for each dilution step. Then, to assess the sensitivity of both assays designed in our study, LOD and LOQ were established following a modified approach of the method outlined in Klymus et al. (2020) (Table S3).

Table 1. Summary of the qPCR results from the positive sites invaded by *C. floridanus* and *C. pseudogracilis* in Ireland. Two eDNA samples (Filter) were collected at each location in Ireland, and all eDNA samples were run in four qPCR replicates.

Name of site sampled	Filter[$]	Dilution[+]	*C. floridanus*		*C. pseudogracilis*	
			qPCR[#]	Cq[£]	qPCR[#]	Cq[£]
River Liffey 2	1	1:1	1/4	39.40	1/4	35.14
Grand Canal 1	1	1:2	3/4	40.10*	1/4	43.24
Lough Ree	2	1:1	0/4	–	4/8	39.85*
Lough Ree	1	1:2	0/4	–	1/4	43.46
Slaney River	1	1:1	0/4	–	1/4	38.77

[$]Filter means how many independent filters were tested positive for each targeted species at a given site.
[+]Dilution "1:1" means undiluted eDNA template, and "1:2" means eDNA template 50% diluted.
[#]qPCR means the number of qPCR replicate positive for the detection of the targeted species.
[£]Cq indicates the Cq values.
*Means that the mean Cq value is indicated.

The following changes were made: the *a priori* function was changed to the log-logistic 2 parameter function, the LOQ CV-threshold was changed to 0.7 for the *C. floridanus* assay and to 10.0 for the *C. pseudogracilis* assay, and the prediction data generation was modified to simulate data on a range better suited for the data. For the latter, the R-package *bigsnpr* was used to enable a logarithmic sequence generating code (Privé et al. 2018). LOD and LOQ were established using R version 4.2.0 (R Core Team 2018) (see Appendix 2 for full R script). A map of the results from qPCR amplifications (see results section) was created using QGIS Desktop 3.16.2 with the GRASS Geographic Information System.

Results

The two sets of primers and probes designed in this study were found to be species specific when tested *in silico* using the NCBI primer blast option (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). Following the machine learning tool developed by Kronenberger et al. (2022) to predict qPCR cross-amplification, our assays were found unlikely to amplify any of the COI fragments from the non-targeted species used in the *in-silico* development (see species list and accession number in Table S1). Specificity tests of the primer/probe assays on DNA extracted from *C. floridanus*, *C. pseudogracilis*, *Gammarus tigrinus*, *G. duebeni*, *G. lacustris*, and *G. pulex*, further revealed no non-specific amplification of these species. Also, the positive and negative controls performed as expected in this study. Following the modified method and protocol outlined in Klymus et al. (2020) (Table S3), the LOQ was established at approximately $2.80 * 10^{-4}$ ng.µL^{-1} for *C. floridanus* and $1.01 * 10^{-4}$ ng.µL^{-1} for *C. pseudogracilis*; and the LOD was established at $9.56 * 10^{-5}$ ng.µL^{-1} for *C. floridanus* and $3.94 * 10^{-5}$ ng.µL^{-1} for *C. pseudogracilis*.

Using four qPCR replicates per eDNA sample, analysis of the samples detected *C. floridanus* and *C. pseudogracilis* in eDNA samples collected in Ireland (Figure 1, Table 1). However, all qPCR replicates of the samples from

Belgium, Netherlands, France, and Norway were negative for both species, i.e., neither species were detected in these countries. The positive eDNA detections from Ireland were further confirmed through Sanger sequencing of the qPCR products. *Crangonyx floridanus* was detected in Kilbride in the River Liffey (see River Liffey 2, Table S2) and in Monasterevin in the Grand Canal (see Grand Canal 1, Table S2), while *C. pseudogracilis* was detected in Kilbride in the River Liffey (see River Liffey 2, Table S2), in Monasterevin in the Grand Canal (see Grand Canal 1, Table S2), in Lough Ree (Table S2), and in the River Slaney (Table S2). *Crangonyx pseudogracilis* was detected in four freshwater systems in Ireland, and *C. floridanus* was also found in two of them. However, it should be noted that *C. pseudogracilis* was detected in only one qPCR replicate with a relatively high Cq value in the Grand Canal. Its presence status at this site could be deemed inconclusive. Furthermore, both species could only be detected at this site in the Grand Canal after diluting the eDNA template, suggesting the presence of PCR inhibitors at this site.

Discussion

In this study we set out to develop and validate two species-specific assays to detect and monitor the two invasive freshwater crangonyctid species, *C. floridanus* and *C. pseudogracilis*. Our aim was first to validate these assays in laboratory conditions and then to monitor these two species in freshwater systems in Ireland where their presence was already known, and to investigate unknown at-risk areas in Belgium, Netherlands, France and Norway. Indeed, both species are known to be present in Irish freshwaters, with recent records and molecular identification (Holmes 1975; Baars et al. 2021). This allowed us to further validate our designed assays with eDNA samples from locations with known presence of these species. Finally, freshwater systems were sampled in France, Belgium, Netherlands and Norway, as *C. pseudogracilis* has previously been found in these countries (Pinkster et al. 1980; Wouters 2002; Galbreath et al. 2010; Spikkeland et al. 2016). However, we had no recent record of this species at the locations sampled in our study (see Appendix 1). We found that both assays designed in our study were species specific and showed relatively similar LOD and LOQ levels. The LOD and LOQ found in our study were consistent with the values observed in similar studies on other freshwater invertebrates (Mauvisseau et al. 2019c, d; Baudry et al. 2021).

The locations sampled in Ireland were chosen following recent sightings of *C. pseudogracilis*, and first records of *C. floridanus* by Baars et al. (2021). Indeed, locations were chosen based on the possibility to detect the newly invasive *C. floridanus* found in the River Liffey and River Barrow and *C. pseudogracilis* (Baars et al. 2021), and to potentially assess their presence in other systems. Here, we detected eDNA traces from *C. floridanus* in the River Liffey and Grand Canal, but not in the River Barrow. The invasive

C. pseudogracilis was detected in the River Liffey, in the Grand Canal, in the Lough Ree, and in the River Slaney. It should be noted that the River Slaney was not investigated in the study from Baars et al. (2021), and this makes it impossible to determine whether the species has been invading the area recently, although our study is the first to record it in this system.

None of the *Crangonyx* species investigated were detected in Belgium, Netherlands, France or Norway. One of the reasons could be that these two species are not yet present in these countries. However, *C. pseudogracilis* has previously been found in Belgium (Wouters 2002), Netherlands (Pinkster et al. 1980), France (Galbreath et al. 2010), and Norway (Spikkeland et al. 2016), so it is most likely that we only sampled systems or sites where the species was not present. Additionally, relatively low volumes of water were filtered in our study due to the high turbidity of the systems at the time of sampling. As a higher volume of water increases the probability of detection (Mächler et al. 2016), we cannot rule out false negative results of *C. pseudogracilis* absence in our study at the sampled sites in Belgium, Netherlands, France, and Norway. Moreover, we discovered an impact of PCR inhibition in the Grand Canal in Ireland, as both species could only be detected after diluting the eDNA templates. Nevertheless, we only observed such effect at this location. It should still be noted that potential inhibition (due to co-extracted compounds or humic acids (Burian et al. 2021), could decrease the probability of detection of both species in Belgium, Netherlands, France, and Norway, but such effects were not tested in our study in the samples collected in these countries. This should however be investigated in future studies, and adequate procedures, such as the use of specific DNA extraction kit, inhibitor removal kits following DNA extraction, or internal positive control should be followed to ensure that inhibition does not lead to false negative records of these species (Mauvisseau et al. 2022). Future work should also investigate the impacts of sampling replication to increase the reliability of eDNA-based detection and consider occupancy modelling to assess the detection probabilities of the species regarding various environmental parameters (Burian et al. 2021; Thalinger et al. 2021). This would allow to increase the "readiness" of our designed eDNA assays from scale 4 to scale 5 as in Thalinger et al. (2021). In contrast to *C. pseudogracilis, C. floridanus* has never been detected in Belgium, Netherlands, France, or Norway, and our results are therefore consistent with these findings. Future research could expand eDNA surveys to an increased number of locations to confirm the absence of *C. floridanus*, as the species has been found outside its natural range only in Japan, England and Ireland. Finally, our designed assays can be used for baseline monitoring and as an early diagnostic tool for the detection of *C. floridanus* in freshwater systems, as well as assess the presence of the previously established *C. pseudogracilis*. This would mitigate the impacts of the invasive *Crangonyx* and avoid potential biodiversity loss in introduced areas (Nagakubo et al. 2011).

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Authors' contribution

Primers and probes development: QM. Laboratory analysis: JB, ASN. eDNA sampling: JRB, QM, RB, CM. Supervision: QM, HdB. Funding acquisition: QM, HdB, JRB. Writing original draft: JB, QM. Review and editing: QM, JB, JRB, ASN, RB, CM, HdB.

References

Baars J-R, Minchin D, Feeley HB, Brekkhus S, Mauvisseau Q (2021) The first record of the invasive alien freshwater amphipod *Crangonyx floridanus* (Bousfield, 1963) (Crustacea: Amphipoda) in two Irish river systems. *BioInvasions Records* 10: 629–635 https://doi.org/10.3391/bir.2021.10.3.13

Bacela-Spychalska K, Wattier RA, Genton C, Rigaud T (2012) Microsporidian disease of the invasive amphipod *Dikerogammarus villosus* and the potential for its transfer to local invertebrate fauna. *Biological Invasions* 14: 1831–1842, https://doi.org/10.1007/s10530-012-0193-1

Bailey RJE, Dick JTA, Elwood RW, MacNeil C (2006) Predatory interactions between the invasive amphipod *Gammarus tigrinus* and the native opossum shrimp *Mysis relicta*. *Journal of the North American Benthological Society* 25: 393–405, https://doi.org/10.1899/0887-3593(2006)25[393:PIBTIA]2.0.CO;2

Bailey SA (2015) An overview of thirty years of research on ballast water as a vector for aquatic invasive species to freshwater and marine environments. *Aquatic Ecosystem Health & Management* 18: 261–268, https://doi.org/10.1080/14634988.2015.1027129

Baudry T, Mauvisseau Q, Goût J-P, Arqué A, Delaunay C, Smith-Ravin J, Sweet M, Grandjean F (2021) Mapping a super-invader in a biodiversity hotspot, an eDNA-based success story. *Ecological Indicators* 126: 107637, https://doi.org/10.1016/j.ecolind.2021.107637

Bódis E, Borza P, Potyó I, Puky M, Weiperth A, Guti G (2012) Invasive mollusc, crustacean, fish and reptile species along the Hungarian section of the River Danube and some connected waters. *Acta zoologica Academiae Scientiarum Hungaricae* 58(Suppl.): 29–55

Brys R, Halfmaerten D, Neyrinck S, Mauvisseau Q, Auwerx J, Sweet M, Mergeay J (2021) Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *Journal of Fish Biology* 98: 399–414, https://doi.org/10.1111/jfb.14315

Burian A, Mauvisseau Q, Bulling M, Domisch S, Qian S, Sweet M (2021) Improving the reliability of eDNA data interpretation. *Molecular Ecology Resources* 21: 1422–1433, https://doi.org/10.1111/1755-0998.13367

Cai W, Harper LR, Neave EF, Shum P, Craggs J, Arias MB, Riesgo A, Mariani S (2022) Environmental DNA persistence and fish detection in captive sponges. *Molecular Ecology Resources* 1755–0998: 13677, https://doi.org/10.1111/1755-0998.13677

Cannizzaro AG, Balding D, Lazo-Wasem EA, Sawicki TR (2020) A new species rises from beneath Florida: molecular phylogenetic analyses reveal cryptic diversity among the metapopulation of *Crangonyx hobbsi* Shoemaker, 1941 (Amphipoda: Crangonyctidae). *Organisms Diversity & Evolution* 20: 387–404, https://doi.org/10.1007/s13127-020-00433-4

Crawford GI (1937) An amphipod, *Eucrangonyx gracilis* S. I. Smith, new to Britain. *Nature* 139: 327, https://doi.org/10.1038/139327b0

Cuthbert RN, Kotronaki SG, Dick JTA, Briski E (2020) Salinity tolerance and geographical origin predict global alien amphipod invasions. *Biology Letters* 16: 20200354, https://doi.org/10.1098/rsbl.2020.0354

Deiner K, Knapp RA, Boiano DM, May B (2013) Increased accuracy of species lists developed for alpine lakes using morphology and cytochrome oxidase I for identification of specimens. *Molecular Ecology Resources* 13: 820–831, https://doi.org/10.1111/1755-0998.12130

DiBacco C, Humphrey DB, Nasmith LE, Levings CD (2012) Ballast water transport of non-indigenous zooplankton to Canadian ports. *ICES Journal of Marine Science* 69: 483–491, https://doi.org/10.1093/icesjms/fsr133

Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T (2015) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS ONE* 10: e0122763, https://doi.org/10.1371/journal.pone.0122763

Dubreuil T, Baudry T, Mauvisseau Q, Arqué A, Courty C, Delaunay C, Sweet M, Grandjean F (2022) The development of early monitoring tools to detect aquatic invasive species: eDNA assay development and the case of the armored catfish *Hypostomus robinii*. *Environmental DNA* 4: 349–362, https://doi.org/10.1002/edn3.260

Galbreath JGMS, Smith JE, Becnel JJ, Butlin RK, Dunn AM (2010) Reduction in post-invasion genetic diversity in *Crangonyx pseudogracilis* (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites? *Biological Invasions* 12: 191–209, https://doi.org/10.1007/s10530-009-9442-3

Gallardo B, Clavero M, Sánchez MI, Vilà M (2016) Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology* 22: 151–163, https://doi.org/10.1111/gcb.13004

Gollasch S, Minchin D, David M (2015) The transfer of harmful aquatic organisms and pathogens with ballast water and their impacts. In: David M, Gollasch S (eds), Global Maritime Transport and Ballast Water Management, Springer Netherlands, Dordrecht, pp 35–58, https://doi.org/10.1007/978-94-017-9367-4_3

Handley LL (2015) How will the 'molecular revolution' contribute to biological recording?: The Molecular Revolution in Biological Recording. *Biological Journal of the Linnean Society* 115: 750–766, https://doi.org/10.1111/bij.12516

Harper LR, Buxton AS, Rees HC, Bruce K, Brys R, Halfmaerten D, Read DS, Watson HV, Sayer CD, Jones EP, Priestley V, Mächler E, Múrria C, Garcés-Pastor S, Medupin C, Burgess K, Benson G, Boonham N, Griffiths RA, Lawson Handley L, Hänfling B (2019) Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia* 826: 25–41, https://doi.org/10.1007/s10750-018-3750-5

Holdich DM, Pöckl M (2007) Invasive crustaceans in European inland waters. In: Gherardi F (ed), Biological invaders in inland waters: Profiles, distribution, and threats, Springer Netherlands, Dordrecht, pp 29–75, https://doi.org/10.1007/978-1-4020-6029-8

Holmes JMC (1975) *Crangonyx pseudogracilis* Bousfield, a freshwater amphipod new to Ireland. *Irish Naturalists' Journal* 18: 225–226

Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of Applied Ecology* 46: 10–18, https://doi.org/10.1111/j.1365-2664.2008.01600.x

Iacarella JC, Dick JTA, Ricciardi A (2015) A spatio-temporal contrast of the predatory impact of an invasive freshwater crustacean. *Diversity and Distributions* 21: 803–812, https://doi.org/10.1111/ddi.12318

Johnson PTJ, Olden JD, Solomon CT, Vander Zanden MJ (2009) Interactions among invaders: community and ecosystem effects of multiple invasive species in an experimental aquatic system. *Oecologia* 159: 161–170, https://doi.org/10.1007/s00442-008-1176-x

Kanada S, Kuranishi RB, Ishiwata S, Tojo K, Shimizu T, Taira H, Satake K (2007) Distribution of an alien species, *Crangonyx floridanus* Bousfield (Crustacea: Amphipoda: Crangonyctidae) in Japan. *Japanese Journal of Limnology (Rikusuigaku Zasshi)* 68: 449–460, https://doi.org/10.3739/rikusui.68.449

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649, https://doi.org/10.1093/bioinformatics/bts199

Kelly NE, Wantola K, Weisz E, Yan ND (2013) Recreational boats as a vector of secondary spread for aquatic invasive species and native crustacean zooplankton. *Biological Invasions* 15: 509–519, https://doi.org/10.1007/s10530-012-0303-0

Kiruba-Sankar R, Praveen Raj J, Saravanan K, Lohith Kumar K, Raymond Jani Angel J, Velmurugan A, Dam Roy S (2018) Invasive species in freshwater ecosystems - Threats to ecosystem services. In: Sivaperuman C, Velmurugan A, Singh AK, Jaisankar I (eds), Biodiversity and Climate Change Adaptation in Tropical Islands, Elsevier, pp 257–296, https://doi.org/10.1016/B978-0-12-813064-3.00009-0

Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2020) Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA* 2: 271–282, https://doi.org/10.1002/edn3.29

Kronenberger JA, Wilcox TM, Mason DH, Franklin TW, McKelvey KS, Young MK, Schwartz MK (2022) eDNAssay: A machine learning tool that accurately predicts QPCR cross-amplification. *Molecular Ecology Resources* 1755–0998: 13681, https://doi.org/10.1111/1755-0998.13681

Lipták B (2013) Non-indigenous invasive freshwater crustaceans (Crustacea: Malacostraca) in Slovakia. *Water Research and Management* 3(3): 12

Lodge DM, Turner CR, Jerde CL, Barnes MA, Chadderton L, Egan SP, Feder JL, Mahon AR, Pfrender ME (2012) Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Molecular Ecology* 21: 2555–2558, https://doi.org/10.1111/j.1365-294X.2012.05600.x

Lynggaard C, Bertelsen MF, Jensen CV, Johnson MS, Frøslev TG, Olsen MT, Bohmann K (2022) Airborne environmental DNA for terrestrial vertebrate community monitoring. *Current Biology* 32: 701–707, https://doi.org/10.1016/j.cub.2021.12.014

MacDonald JA, Roudez R, Glover T, Weis JS (2007) The invasive green crab and Japanese shore crab: behavioral interactions with a native crab species, the blue crab. *Biological Invasions* 9: 837–848, https://doi.org/10.1007/s10530-006-9085-6

Mächler E, Deiner K, Spahn F, Altermatt F (2016) Fishing in the water: Effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. *Environmental Science & Technology* 50: 305–312, https://doi.org/10.1021/acs.est.5b04188

Mächler E, Deiner K, Steinmann P, Altermatt F (2014) Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. *Freshwater Science* 33: 1174–1183, https://doi.org/10.1086/678128

Martínez J, Adarraga I (2008) First record of invasive caprellid *Caprella scaura* Templeton, 1836 sensu lato (Crustacea: Amphipoda: Caprellidae) from the Iberian Peninsula. *Aquatic Invasions* 3: 165–171, https://doi.org/10.3391/ai.2008.3.2.6

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M (2019a) Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Scientific Reports* 9: 580, https://doi.org/10.1038/s41598-018-37001-y

Mauvisseau Q, Davy-Bowker J, Bryson D, Souch GR, Burian A, Sweet M (2019b) First detection of a highly invasive freshwater amphipod *Crangonyx floridanus* (Bousfield, 1963) in the United Kingdom. *BioInvasions Records* 8: 1–7, https://doi.org/10.3391/bir.2019.8.1.01

Mauvisseau Q, Davy-Bowker J, Bulling M, Brys R, Neyrinck S, Troth C, Sweet M (2019c) Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. *Scientific Reports* 9: 14064, https://doi.org/10.1038/s41598-019-50571-9

Mauvisseau Q, Harper LR, Sander M, Hanner RH, Kleyer H, Deiner K (2022) The multiple states of environmental DNA and what is known about their persistence in aquatic environments. *Environmental Science & Technology* 56: 5322–5333, https://doi.org/10.1021/acs.est.1c07638

Mauvisseau Q, Kalogianni E, Zimmerman B, Bulling M, Brys R, Sweet M (2020) eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species. *Environmental DNA* 2: 601–613, https://doi.org/10.1002/edn3.92

Mauvisseau Q, Tönges S, Andriantsoa R, Lyko F, Sweet M (2019d) Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems. *Management of Biological Invasions* 10: 461–472, https://doi.org/10.3391/mbi.2019.10.3.04

Minamoto T, Hayami K, Sakata MK, Imamura A (2019) Real-time polymerase chain reaction assays for environmental DNA detection of three salmonid fish in Hokkaido, Japan: Application to winter surveys. *Ecological Research* 34: 237–242, https://doi.org/10.1111/1440-1703.1018

Moorhouse TP, Macdonald DW (2015) Are invasives worse in freshwater than terrestrial ecosystems? *WIREs Water* 2: 1–8, https://doi.org/10.1002/wat2.1059

Morino H, Kusan H, Holsinger JR (2004) Description and distribution of *Crangonyx floridanus* (Crustacea : Amphipoda : Crangonyctidae) in Japan, an introduced freshwater amphipod from North America. *Contributions from the Biological Laboratory, Kyoto University* 29(4): 371–381

Nagakubo A, Sekiné K, Tanaka Y, Kuranishi RB, Kanada S, Tojo K (2011) Rapid expansion of the distributional range and the population genetic structure of the freshwater amphipod *Crangonyx floridanus* in Japan. *Limnology* 12: 75–82, https://doi.org/10.1007/s10201-010-0323-3

Nordstrom B, Mitchell N, Byrne M, Jarman S (2022) A review of applications of environmental DNA for reptile conservation and management. *Ecology and Evolution* 12: e8995, https://doi.org/10.1002/ece3.8995

Novitsky RA, Son MO (2016) The first records of Marmorkrebs [*Procambarus fallax* (Hagen, 1870) f. *virginalis*] (Crustacea, Decapoda, Cambaridae) in Ukraine. *Ecologica Montenegrina* 5: 44–46, https://doi.org/10.37828/em.2016.5.8

Panov VE, Krylov PI, Riccardi N (2004) Role of diapause in dispersal and invasion success by aquatic invertebrates. *Journal of Limnology* 63: 56, https://doi.org/10.4081/jlimnol.2004.s1.56

Pinkster S, Dieleman J, Platvoet D (1980) The present position of *Gammarus tigrinus* Sexton, 1939, in the Netherlands, with the description of a newly discovered amphipod species, *Crangonyx pseudogracilis* Bousfield, 1958 (Crustacea, Amphipoda). *Bulletin Zoologisch Museum* 7(4): 33–38

Pintor LM, Sih A (2009) Differences in growth and foraging behavior of native and introduced populations of an invasive crayfish. *Biological Invasions* 11: 1895–1902, https://doi.org/10.1007/s10530-008-9367-2

Privé F, Aschard H, Ziyatdinov A, Blum MGB (2018) Efficient analysis of large-scale genome-wide data with two R packages: bigstatsr and bigsnpr. *Bioinformatics* 34: 2781–2787, https://doi.org/10.1093/bioinformatics/bty185

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) REVIEW: The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* 51: 1450–1459, https://doi.org/10.1111/1365-2664.12306

Rodríguez-Rey M, Consuegra S, Börger L, Garcia de Leaniz C (2019) Improving Species Distribution Modelling of freshwater invasive species for management applications. *PLoS ONE* 14: e0217896, https://doi.org/10.1371/journal.pone.0217896

Rourke ML, Fowler AM, Hughes JM, Broadhurst MK, DiBattista JD, Fielder S, Wilkes Walburn J, Furlan EM (2022) Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA* 4: 9–33, https://doi.org/10.1002/edn3.185

Sepulveda AJ, Nelson NM, Jerde CL, Luikart G (2020) Are environmental DNA methods ready for aquatic invasive species management? *Trends in Ecology & Evolution* 35: 668–678, https://doi.org/10.1016/j.tree.2020.03.011

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Biological Sciences* 103: 12115–12120, https://doi.org/10.1073/pnas.0605127103

Spikkeland I, Olsen KM, Kinsten B, Kjellberg G (2016) The freshwater amphipod *Crangonyx pseudogracilis* found in Norway. *Fauna* 69(1): 24–34

Strecker AL, Arnott SE (2008) Invasive predator, *Bythotrephes*, has varied effects on ecosystem function in freshwater lakes. *Ecosystems* 11: 490–503, https://doi.org/10.1007/s10021-008-9137-0

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. *Molecular Ecology* 21: 1789–1793, https://doi.org/10.1111/j.1365-294X.2012.05542.x

Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS, Bruce K (2021) A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA* 3: 823–836, https://doi.org/10.1002/edn3.189

Thomsen PF, Willerslev E (2015) Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* 183: 4–18, https://doi.org/10.1016/j.biocon.2014.11.019

Tojo K, Tanaka Y, Kuranishi RB, Kanada S (2010) Reproductive biology and adaptability of the invasive alien freshwater amphipod *Crangonyx floridanus* (Crustacea: Amphipoda, Crangonyctidae). *Zoological Science* 27: 522–527, https://doi.org/10.2108/zsj.27.522

Turner CR, Uy KL, Everhart RC (2015) Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation* 183: 93–102, https://doi.org/10.1016/j.biocon.2014.11.017

Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D, Johnsen S, Steffensen JP, Bennike O, Schwenninger J-L, Nathan R, Armitage S, de Hoog C-J, Alfimov V, Christl M, Beer J, Muscheler R, Barker J, Sharp M, Penkman KEH, Haile J, Taberlet P, Gilbert MTP, Casoli A, Campani E, Collins MJ (2007) Ancient biomolecules from deep Ice cores reveal a forested southern Greenland. *Science* 317: 111–114, https://doi.org/10.1126/science.1141758

Williams PJ, Floyd TA, Rossong MA (2006) Agonistic interactions between invasive green crabs, *Carcinus maenas* (Linnaeus), and sub-adult American lobsters, *Homarus americanus* (Milne Edwards). *Journal of Experimental Marine Biology and Ecology* 329: 66–74, https://doi.org/10.1016/j.jembe.2005.08.008

Wouters K (2002) On the distribution of alien non-manne and estuarine macro-crustaceans in Belgium. *Biologie* 72: 119–129

Xu CCY, Yen IJ, Bowman D, Turner CR (2015) Spider web DNA: A new spin on noninvasive genetics of predator and prey. *PLoS ONE* 10: e0142503, https://doi.org/10.1371/journal.pone.0142503

Zhang J (1997) Systematics of the freshwater amphipod genus *Crangonyx* (Crangonyctidae) in North America. Doctoral dissertation, Old Dominion University

Web sites and online databases

R Core Team (2018) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/ (accessed 2 June 2022)

Supplementary material

The following supplementary material is available for this article:

Appendix 1. Extraction protocols and other information related to the eDNA samples from each country.
Appendix 2. R script to calculate Limit of Detection for eDNA assay.
Table S1. Overview of targeted, potentially co-occurring or closely related species and their associated GenBank accession numbers used during the development and validation of both assays in this study.
Table S2. Summary information of the eDNA samples from France, Ireland, Norway, Belgium and in The Netherlands.
Table S3. Raw data generated to establish the LOD and LOQ.
Figure S1. Figure showing mismatches between the species-specific primers and probe and the targeted COI sequences of *Crangonyx pseudogracilis* and other closely related or potentially co-occurring species.
Figure S2. Figure showing mismatches between the species-specific primers and probe and the targeted COI sequences of *Crangonyx floridanus* and other closely related or potentially co-occurring species.
Figure S3. Map showing the locations of the sampling sites in Europe. For additional details see Table S2.