# Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA

Chloe Victoria **Robinson**[1], Tamsyn M. **Uren Webster**[1], Joanne **Cable**[2], Joanna **James**[2,3], Sofia **Consuegra**[1]

[1]Swansea University, Singleton Park, Swansea, SA2 8PP, Wales, UK

[2] School of Biosciences, Cardiff University, Cardiff, CF10 3AT, Wales, UK

[3]Environment Agency, Bromholme Lane, Brampton, PE28 4NE, UK

Key words: eDNA, *Pacifastacus leniusculus*, *Austropotamobius pallipes*, AIS, crayfish, conservation, *Aphanomyces astaci*, detect, qPCR-HRM.

## ABSTRACT

Aquatic Invasive Species (AIS) are important vectors for the introduction of novel pathogens which can, in turn, become drivers of rapid ecological and evolutionary change, compromising the persistence of native species. Conservation strategies rely on accurate information regarding presence and distribution of AIS and their associated pathogens to prevent or mitigate negative impacts, such as predation, displacement or competition with native species for food, space or breeding sites. Environmental DNA is increasingly used as a conservation tool for early detection and monitoring of AIS. We used a novel eDNA high-resolution melt curve (HRM) approach to simultaneously detect the UK endangered native crayfish (*Austropotamobius pallipes*), the highly invasive signal crayfish (*Pacifastacus leniusculus*) and their dominant pathogen, *Aphanomyces astaci*, (causative agent of crayfish plague). We validated the approach with laboratory and field samples in areas with known presence or absence of both crayfish species as well as the pathogen, prior to the monitoring of areas where their presence was unknown. We identified the presence of infected signal crayfish further upstream than previously detected in an area where previous intensive eradication attempts had taken place, and the coexistence of both species in plague free catchments. We also detected the endangered native crayfish in an area where trapping had failed. With this method, we could estimate the distribution of native and invasive crayfish and their infection status in a rapid, cost effective and highly sensitive way, providing essential information for the development of conservation strategies in catchments with populations of endangered native crayfish.

## INTRODUCTION

Invasive non-native species have become important drivers of global environmental change (Vitousek et al. 1996), although the importance of their impacts on biodiversity remains controversial (Russell and Blackburn 2017). Their spread has been favoured by human-mediated activities (Crowl et al. 2008) in addition to natural dispersal, and, as a consequence have also become common vehicles for the introduction of novel pathogens (Randolph and Rogers 2010). Invasive non-native species extend the geographic range of the pathogens they carry and facilitate host-switching (Peeler et al. 2011). In turn, pathogens play an important role in the evolution of communities but can also threaten the survival of native populations (Altizer et al. 2003). Co-introductions of parasites with non-native hosts are common; invasive species may bring novel infectious diseases that can infect native competitors, but can also act as hosts and effective dispersers for native diseases (Strauss et al. 2012). Invasive pathogens can have devastating effects on vulnerable native hosts, as their virulence tends to be higher than in the non-native species (Lymbery et al. 2014). Such pathogens seem particularly frequent in freshwater species, potentially reflecting the high susceptibility of freshwater ecosystems to non-native invasions (Moorhouse and Macdonald 2015). Thus, early detection of both non-native hosts and parasites is critical for the control and management of the impacts caused by introduced diseases.

Detection of non-native species often occurs when populations have already established, spread from original source and altered the local environment (Vander Zanden et al. 2010; Zaiko et al. 2014). This is particularly the case in aquatic environments, where juveniles or larvae at the initial stages of introduction often have a patchy distribution, are difficult to identify using morphological techniques, and are easily missed by monitoring programmes (Pochon et al. 2013). Early detection is needed to make management actions such as eradication and control of invasive species more efficient and/or effective (Lodge et

al. 2016) and as such is becoming fundamental for the management and control of aquatic invasive species (AIS; Vander Zanden et al. 2010). Analysis of environmental DNA (eDNA), i.e. free DNA molecules released from sources such as faeces, skin, urine, blood or secretions of organisms, is proving increasingly useful for detecting species that are difficult to identify and locate by more traditional and time-consuming methods (Biggs et al. 2015), such as endangered species (Dejean et al. 2011) and AIS at the early stages of their introduction (Bohmann et al. 2014; Dejean et al. 2012). Although still a relatively new tool, eDNA is becoming widely used for conservation (Biggs et al. 2015; Laramie et al. 2015; Spear et al. 2015; Thomsen and Willerslev 2015) and protocols are being refined to increase its accuracy and reliability (Goldberg et al. 2016; Wilson et al. 2016). Quantitative PCR (qPCR) is commonly used to target particular species in eDNA samples (e.g. (Ficetola et al. 2008; Thomsen et al. 2012) and, coupled with *in vitro* controls and amplicon sequencing, has proved a reliable method for the detection of invasive and endangered aquatic species (Klymus et al. 2015; Spear et al. 2015). In addition, qPCR is widely used to detect infectious agents in environmental samples (Guy et al. 2003), and can be particularly useful for the early detection of aquatic pathogens which can be introduced simultaneously with non-native species (Ganoza et al. 2006; Strand et al. 2014). High Resolution Melting (HRM) analysis is a qPCR-based method which facilitates identification of small variations in nucleic acid sequences by differences in the melting temperature of double stranded DNA depending on fragment length and sequence composition (Ririe et al. 1997). Analysis of HRM curves has been widely used for SNP genotyping as a fast method to discriminate species (Yang et al. 2009), including natives and invasives (Ramón-Laca et al. 2014). HRM has the potential for being used in AIS identification, including aquatic invasive pathogens, but it has not yet been applied to their detection from eDNA samples. We have used this method to investigate the distribution of the invasive signal crayfish (*Pacifastacus leniusculus*), carrier of the crayfish

71 plague agent (*Aphanomyces astaci*) which is highly infective for native species (e.g.
72 *Austropotamobius pallipes*), and the potential coexistence between native and invasive
73 crayfish in UK populations.

74 Invasive non-native crayfish have been globally introduced, mainly for human
75 consumption, and are known to seriously impact native ecosystems through predation,
76 competition, disease transmission and hybridisation (e.g. Lodge et al. 2012). In Europe, non-
77 indigenous crayfish mostly of North American origin have outnumbered their native
78 counterparts in much of their range and represent one of the main threats to their persistence
79 (Holdich et al. 2009). The distribution and abundance of native European crayfish species has
80 been strongly influenced by high mortality rates associated with contracting crayfish plague
81 (Schrimpf et al. 2012) through the introduction of North American freshwater crayfish
82 around 1850 (Alderman 1996). *P. leniusculus* was one of the first non-native species
83 introduced to Europe and in the UK is displacing the native crayfish (*A. pallipes*) which has
84 been classified as endangered in the UK (IUCN 2017). Its success has been attributed to
85 preadaptation, niche plasticity, the aggressive nature of the species (Chapple et al. 2012;
86 Pintor et al. 2008) and/or the competitive advantage provided by the crayfish plague (Bubb,
87 Thom, and Lucas 2006; Dunn et al. 2012; Edgerton et al. 2004; Griffiths, Collen and
88 Armstrong, 2004).

89 By using a novel approach to simultaneously identify both AIS and their major
90 associated pathogens, we analysed the distribution of the highly invasive signal crayfish (*P.*
91 *leniusculus*), the native crayfish (*A. pallipes*) and the crayfish plague pathogen (*A. astaci*) in
92 areas where the presence of the signal crayfish is severely impacting the native populations,
93 to identify potential areas of coexistence and refugia for the native species. We expected to
94 find coexisting populations of both species more likely in locations where the crayfish plague
95 has been historically and continually absent.

## MATERIALS AND METHODS

### *EX SITU* OPTIMISATION OF eDNA METHODS

In order to optimise eDNA protocols an *ex-situ* pilot experiment was conducted by placing individual *P. leniusculus* in three isolated tanks, each with 2 L of water. After 24 hours, they were removed and two 15 mL water samples were taken from each tank. The sampling was repeated 24 and 48 hours after removal. Two ultrapure water blanks and four tank blanks (with no crayfish in) were also taken as controls during each sampling period. Immediately after collection, a standard method of preserving and extracting eDNA was applied by the addition of 33 mL of absolute ethanol and 1.5 mL of 3M sodium acetate to samples and subsequent storage at -20$^{o}$C for a minimum of 24 hours before DNA extraction (Ficetola et al. 2008). To recover precipitated DNA, samples were centrifuged to create a DNA pellet. The supernatant was discarded and the remaining pellet was air-dried before being subjected to DNA extraction. Extraction blanks consisting of ultrapure water in place of sampled water and tank blanks were used to test for any cross-contamination of the samples. Similarly, nine 15 mL water samples were taken, along with a system blank, at a local hatchery containing a population of *A. pallipes*, to test detection levels of native crayfish in aqueous eDNA samples.

### STUDY POPULATIONS AND eDNA SAMPLE COLLECTION

We sampled six locations in the River Wye catchment and seven additional sites in the River Taff catchment, both in Wales, UK (Figure 1a-c), as well as a total of 29 sites in two catchments from Southern England, the Itchen and Medway rivers (Figure 1c; Table 1), all of them introduced c.1970. Records of the introduction of signal crayfish in Europe are very limited, but some evidence suggests that between 1976 and 1978 around 150,000 juvenile

121 signal crayfish were introduced into Britain and other European countries from a hatchery in
122 Simontorp, Sweden, which originally imported them from Lake Tahoe in California and
123 Nevada, USA, in 1969 (Holdich and Lowery, 1988). After the Simontorp introductions,
124 crayfish began to be imported directly from different American hatcheries (Holdich and
125 Lowery, 1988), suggesting that the current populations could have different origins, and
126 potentially initial infection status.

127 Welsh locations were selected based upon data from CrayBase (James et al. 2014a);
128 two of the locations supported *A. pallipes* populations, with no evidence of *P. leniusculus*
129 presence, three locations only had populations of *P. leniusculus* and the remaining eight
130 locations could potentially have both *P. leniusculus* and *A. pallipes* or neither species, but
131 their status was uncertain as these had not been previously monitored. Two out of the three *P.*
132 *leniusculus* confirmed sites were known to contain *A. astaci* infected crayfish (James et al.
133 2017).

134 In the river Medway, *P. leniusculus* was thought to inhabit the upper catchment but
135 the crayfish status downstream was unknown, while in the river Itchen *A. pallipes* was
136 assumed to be present throughout most of the upper catchment and *P. leniusculus* had been
137 recorded in few sites both upstream and downstream of *A. pallipes* presence (Rushbrook
138 2014); Table 1). The infection status of both the Medway and Itchen crayfish populations was
139 unknown.

140 Each site was subdivided into three sampling sites (upstream, midstream and
141 downstream), separated where possible by ca. 500 m, to increase the area sampled. Between
142 three and nine 15 mL water samples were taken from each sampling site simultaneously. All
143 samples were collected ca. 1 m beneath the surface for ponds and in shallow areas of low
144 flow streams and preserved as for the *ex-situ* experiment. Negative controls consisting of

ultrapure water in place of river/pond water were taken before and after sampling, at each sampling site. Temperature, weather conditions, amount of shade cover, flow rate and pH were measured at each site (Table 1). Footwear was washed with Virkon™ and equipment disinfected with bleach between samplings to prevent the possible spread of *A. astaci* spores and DNA contamination between sites. All Wye sites which indicated presence of either crayfish species based on initial qPCR results were re-sampled the following year to assess reproducibility of positive amplifications at the sites (Table 1). To estimate the current presence of both host species, 25 standard TRAPPY™ crayfish traps (500 x 200 x 57 mm; NRW Permit Reference: NT/CW081-B-797/3888/02) were set following standard guidelines for trapping crayfish (DEFRA 2015). Traps were baited with halibut pellets and set at all of the eDNA sample sites and left for 24-48 hour, and 24 hour checks were conducted. Three 15 mL water samples were taken downstream of traps (or around the trap for still water bodies) which had successfully trapped crayfish, as a control of crayfish eDNA detectability in the river. Crayfish were collected and euthanised by freezing at -20 ºC (Cooper 2011). Environmental data was recorded at each site as detailed above (Table 2).

Positive controls for eDNA screening consisted of 15 tissue samples from *P. leniusculus* individuals (pooled tail fan and soft cuticle) from three different source populations (Gavenny, Bachowey and Mochdre), part of a previous study within close proximity to eDNA sampling sites within the Bachowey and Duhonw catchments (James et al. 2017), and 12 *A. pallipes* individuals (first carapace moults and mortalities preserved in 100% ethanol) from two different locations in the UK (Cynrig Hatchery, Brecon and Bristol Zoo).

qPCR PRIMER DESIGN

169 Crayfish specific primers were designed using Primer3 software, tested *in silico* using
170 Beacon Primer Designer (ver. 2.1, PREMIER Biosoft), and checked for cross-amplification
171 using NCBI Primer-BLAST (Ye et al. 2006). The primer pair was designed to be
172 complementary to both the signal crayfish and native white-clawed crayfish (ApalPlen16SF:
173 5'-AGTTACTTTAGGGATAACAGCGT-3' and ApalPlen16SR: 5'-
174 CTTTTAATTCAACATCGAGGTCG-3'), to allow the amplification of an 83bp fragment of
175 the 16S mtDNA gene (Data in brief Figure 1). The primers were assessed *in vitro* using
176 positive control tissue (crayfish tail fan clips and moults) from 15 different signal and white-
177 clawed crayfish individuals. DNA was extracted using Qiagen® DNeasy Blood and Tissue
178 Kit (Qiagen, UK), eluted in 100 µl, and amplified in end-point PCR using the following
179 ApalPlen16S protocol: 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 30 s, 61.5 ºC for
180 30 s and 72 ºC for 45 s with a final elongation step of 72 ºC for 10 min. All amplified PCR
181 products were checked for the correct amplicon size using a 2% agarose gel electrophoresis.
182 Primers were also tested on tissue samples from a second invasive crayfish species
183 established in the UK, the virile crayfish (*Orconectes* cf. *virilis*), and against a related species
184 commonly found in the same environment, the freshwater shrimp (*Gammarus* sp.) to check
185 for non-specific amplification.

186 DNA from the *ex-situ* eDNA samples for *P. leniusculus* and *A. pallipes* were
187 extracted using Qiagen® DNeasy Blood and Tissue Kit (Qiagen, UK), eluted in 100 µl, and
188 amplified with ApalPlen16S primers. PCR products were run in a 2% agarose gel to check
189 for correct amplicon size against positive controls (extracted crayfish tail clip), purified and
190 analysed using Sanger Sequencing on an ABI Prism 277 DNA sequencer. Resulting
191 sequences were aligned using BioEdit v. 5.0.9 (using the ClustalW program) and inputted to
192 BLAST (Ye et al. 2006) to confirm the species identity.
193

194 qPCR-HRM OPTIMISATION

195 Specific *in vitro* testing of RT-qPCR-HRM analysis was performed for both *P. leniusculus*
196 and *A. pallipes* crayfish samples to ensure that each species could be identified based on their
197 specific differential PCR product melt temperatures. Annealing temperature for ApalPlen16S
198 primers was optimised at 61.5 ºC and resulting efficiency values at this temperature for both
199 species were 92.0 and 93.8% for *P. leniusculus* and *A. pallipes*, respectively. For
200 optimisation, the ApalPlen16S-qPCR cycling protocol began with 15 min of denaturation at
201 95 ºC, followed by 40 cycles of 95 ºC for 10 s and 61.5 ºC for 30 s. A HRM step was applied
202 to the end of RT-qPCR reactions, ranging from 55 ºC to 95 ºC in 0.1 ºC increments to assess
203 the consistency of amplicon melt temperature (tm) for both crayfish species. Limit of
204 detection (LOD) and limit of quantification (LOQ) were determined through running a
205 dilution series ranging from 5 ng/µl to 5 x $10^{-7}$ ng/µl, using DNA pools for both species.
206 HRM analysis was conducted on a minimum of 12 and a maximum of 15 individuals from
207 several *P. leniusculus* and *A. pallipes* populations to account for any potential intraspecific
208 variation in qPCR product tm (Table 3). qPCR-HRM analysis was undertaken comparing two
209 master mixes, SYBR® Green (Bio-Rad, UK) and SsoFast™ EvaGreen® (Bio-Rad, UK),
210 assessing consistency and reproducibility of both with relation to melt curve profiles (Table
211 3). To assess ability to detect both crayfish species in the same reaction, equal volumes of *P.*
212 *leniusculus* and *A. pallipes* DNA were pooled together from ten different individuals of both
213 species at various concentration ratios (ranging from 50:50 to 10:90).

214 Once the *in vitro* testing was complete for positive controls, further testing was
215 undertaken for the eDNA samples collected in the *ex-situ* study to ensure that the primers
216 would amplify environmental DNA samples and to assess the minimum levels of detection of
217 eDNA samples.

218

## MULTIPLEX OPTIMISATION

For the *A. astaci* multiplex assay, optimisation of primer quantity and concentration was undertaken by combining the two sets of primers (ApalPlen16S and AphAstITS; (Vrålstad et al. 2009) at starting concentrations between 1 µM and 20 µM. Equal concentrations of each set of primers at 5 µM produced the most efficient co-amplification for both sets of primers, with poor amplifications resulting in concentrations from 1 to 4 µM and above 6 µM starting concentration. Uninfected crayfish DNA controls were obtained through extraction of a tail fan clip from non-infected individuals and *A. astaci*-positive samples were obtained from a previous study by Cardiff University (James et al. 2017), where an infected crayfish tail fan clip, melanised soft cuticle and walking leg tissue were pooled together and DNA extracted for *A. astaci* screening.

The final optimised multiplex qPCR reactions were carried out in a final volume of 10 µl, which contained 2 µl 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus ROX (Soils Biodyne, Estonia), 0.4 µl of primer mix (5 µM), 1 µl template DNA at 5 ng/µl and 6.6 µl of ultrapure water. The amplification was carried out using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK). The PCR protocol was as follows: once cycle of initial activation at 95 ºC for 12 min, followed by 40 cycles of 95 ºC for 15 s, 61.5 ºC for 20 s and 72 ºC for 20 s. After the PCR reaction, a melt curve program was set, which ran from 65 ºC to 95 ºC by raising 1 ºC for 10 s each step. The resulting curve was then used to assess the presence/absence of *A. astaci* and target crayfish species DNA based on the species-specific melting temperatures of the DNA product (*A. astaci* = 82.9 ºC; *P. leniusculus* = 75.9 ± 0.2ºC and *A. pallipes* = 76.6 ± 0.2ºC) which were identified during optimization of the multiplex assay.

## eDNA *IN SITU* ANALYSES

eDNA extraction from 407 field samples (Table 1) was performed using Qiagen® DNeasy Blood and Tissue Kit (Qiagen, UK), following the manufacturer's instructions, apart from a reduction in the elution volume from a single elution step of 200 µl to two elution steps of 50 µl to maximise DNA yield. DNA extractions took place in a dedicated eDNA area within an extraction cabinet, equipped with a UV light and a flow-through air system to minimise chances of contamination. Extractions were conducted wearing eDNA-dedicated laboratory coat, face mask and gloves. Samples were amplified in triplicate in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK), in 10 µl reactions consisting of 5 µl SsoFast™ EvaGreen® Supermix (Bio-Rad, UK), 0.25 µl each ApalPlen16SF and ApalPlen16SR, 3.5 µl HPLC water and 2 µl extracted DNA. Amplifications were carried out in triplicate using standard ApalPlen16s-qPCR protocol as described above and only samples which amplified consistently in at least two replicates at the target DNA product tm (either 73.9 ± 0.2 or 74.8 ± 0.2 $^{o}$C), with a melt rate above 200 -d(RFU)/dT were considered to be a positive result. qPCR reactions were carried out in a separate room to eDNA extractions under a PCR hood with laminar flow. Two positive controls per species were added to each plate once all the eDNA samples were loaded and sealed to prevent false positives in the eDNA samples. Two amplification negative controls consisting of HPLC water and two extraction negative controls were also added in the same well location on each plate test for contamination in eDNA samples.

A subset of positive field samples, along with a positive control for each crayfish species were re-amplified using end-point PCR, purified and cloned into pDrive plasmid cloning vector (Qiagen PCR Plus Cloning Kit, Qiagen, UK). Three to nine clones per sample were sequenced using T7 and SP6 primers on an ABI Prism 377 sequencer.

## RESULTS

## *EX-SITU* OPTIMISATION

Optimisation of eDNA protocols was carried out *ex-situ* by placing individual *P. leniusculus* in isolated tanks for 24 hours and sampling water from those tanks 24 and 48 hours after removal. Reference DNA from the *ex-situ* study was successfully extracted and amplified in triplicate from *P. leniusculus* and *A. pallipes* positive controls and species confirmed by Sanger Sequencing of the 83bp fragment of the 16S mtDNA. DNA from signal crayfish was detected in all water samples taken at different time points from the *ex-situ* study. eDNA concentrations marginally decreased overtime and correlated with Cq values for *ex-situ* samples in qPCR amplifications (Data in brief: Figure 2; Figure 5B; Table 3). DNA from native crayfish was also amplified in all nine water samples from the reference hatchery. No amplification bands were present in any of the negative controls (tank, extraction and amplification).

## CRAYFISH DETECTION LIMITS

The results of the qPCR optimisation indicated that the limit of detection (LOD) of both *P. leniusculus* and *A. pallipes* DNA was 0.005 ng/µl, after a 10-fold dilution series. The detection threshold for amplification of positive control DNA used for optimisation from both species was between 16 and 28 cycles, and the melting temperatures (tm) of the DNA products were consistent for both *P. leniusculus* and *A. pallipes*, with no overlap between the two species (Table 3). SsoFast™ EvaGreen® multiplex master mix performed more consistently than the SYBR® Green master mix, with a lower standard deviation for average tm, average peak height, average start melt temperature and average end melt temperature (Table 3; Data in brief: Figure 3; Table 1). Results of the qPCR analysis of mixed proportions of *P. leniusculus* and *A. pallipes* DNA confirmed that it is possible to discriminate between positive amplifications of eDNA for single crayfish species vs. mixed crayfish species (*P.*

294 *leniusculus* and *A. pallipes*). Diagnostic peaks in early product melt temperatures were
295 present for all amplifications containing 90:10 to 50:50 ratios of *P. leniusculus: A. pallipes*
296 DNA (Figure 2; Data in brief: Figure 5A; Table 2).
297
298 SIMULTANEOUS DETECTION OF CRAYFISH AND *APHANOMYCES ASTACI*
299 The multiplex assay for simultaneous crayfish and *A. astaci* detection resulted in two
300 products with an average tm of 75.9 ± 0.2 ºC for *P. leniusculus* (or 76.6 ± 0.2 ºC for *A.*
301 *pallipes*; four individuals) and 82.9 ºC for *A. astaci.* DNA controls from four *A. astaci*-
302 infected *P. leniusculus* individuals (INF 1 – INF 4) were successfully amplified with two
303 products of the corresponding temperatures. Amplification of uninfected *P. leniusculus* DNA
304 resulted in a single product with tm of 75.9 ± 0.2 ºC (Figure 2; Data in brief: Figure 6; Table
305 4).
306
307 CRAYFISH SPECIES DISTRIBUTION AND INFECTION STATUS

308 For Welsh sites, crayfish trapping confirmed the presence of *P. leniusculus* (11 caught across
309 3 different sites; Table 2) in positive sites, whereas no *A. pallipes* were caught, despite visual
310 confirmation of the species upon collecting traps. *P. leniusculus* eDNA was successfully
311 detected around each of the three traps in the reservoir (Data in brief: Figure 7; Table 5).
312 qPCR detected *P. leniusculus* eDNA at all three confirmed sites for the species and *A.*
313 *pallipes* eDNA was detected within the confirmed tributary for the species (Data in brief:
314 Figure 8A-B; Table 6). Additionally, *P. leniusculus* eDNA was detected in one of the
315 unknown crayfish status sites in the river Taff whereas there was no positive detection of *A.*
316 *pallipes* in any of the other the sites with unknown presence of the species (Figure 3, Table
317 4).

In both the Medway and Itchen there was evidence of *P. leniusculus* and *A. pallipes* coexisting in two sampling sites (Data in brief: Figure 9; Table 7). One site in the Medway was positive for both crayfish species over the two-year sampling period and one site in the Itchen was also positive for both species in the single sampling event carried out. Both *A. pallipes* and *P. leniusculus* were also detected in the Medway and Itchen in separate areas (*A. pallipes*: Medway (2 sites), Itchen (4 sites); *P. leniusculus*: Medway (3 sites), Itchen (9 sites).

*A. astaci* was confirmed in all sites in the river Bachowey, resulting in two products with melt peaks at 75.9 ± 0.2 and 82.9ºC for the signal crayfish and plague agent respectively (Data in brief: Figure 8C; Table 6). All other sites positive for *P. leniusculus* or *A. pallipes* were negative for *A. astaci,* which was not detected in the rivers Medway or Itchen, despite the coexistence of both crayfish species (Figure 4).

A subset of five positive amplifications was selected (one for *A. pallipes* and four for *P. leniusculus*) to confirm species identity by cloning and sequencing. Out of 36 successfully transformed clones for the field samples of *P. leniusculus* (nine for each sample), between two and nine clone sequences per sample matched 100% with *P. leniusculus* on BLAST (Ye et al. 2006); remaining clones were a product of non-specific amplification. For *A. pallipes* field samples, two out of 3 clones from the positive field sample matched 100% for *A. pallipes*. All six positive control clones matched 100% with respective crayfish species (*P. leniusculus*/*A. pallipes*).

## DISCUSSION

By using a novel multiplex approach we could simultaneously detect the presence of the endangered white clawed crayfish and the highly invasive North American signal crayfish within a catchment that was free of crayfish plague. In contrast, we did not detect any native

342 crayfish or coexistence of both species in tributaries where the pathogen was identified. A
343 common impact of invasive species on native populations is the transmission of pathogens.
344 Many non-native species not only introduce novel pathogens (Miaud et al. 2016) but also act
345 as non-clinical carriers, facilitating their dispersal (Andreou et al. 2012). In this way,
346 pathogens can act as biological weapons that allow invasive species to outcompete their
347 native counterparts (Vilcinskas 2015), as in the case of the UK native crayfish, highly
348 susceptible to the plague carried out, mostly asymptomatically, by the invasive signal
349 crayfish (Andreou et al. 2012). As highlighted in the principles adopted by the Convention on
350 Biological Diversity on invasive species, prevention and early detection should represent the
351 priority responses to invasive species to allow for rapid response and more cost-effective
352 removal when possible (Simberloff et al. 2013) and our study is the first one to combine
353 eDNA and HRM for early detection of novel pathogens carried by non-native species, being
354 particularly relevant for management and conservation in relation to aquatic biological
355 invasions.

356 The causal agent of crayfish plague, *A. astaci*, is considered one of the primary causes
357 for the extirpation of native crayfish populations across Europe (Alderman et al. 1990; Dunn
358 et al. 2009). Attempts to eradicate established populations of *P. leniusculus* and other
359 invasive non-native crayfish have been largely unsuccessful and costly (Dougherty et al.
360 2016; Kirjavainen and Sipponen 2004; Peay 2009; Sandodden and Johnsen 2010) and
361 increasing emphasis is being placed on early detection of non-native crayfish, rather than on
362 eradication of established populations (Freeman et al. 2010; Gherardi et al. 2011; James et al.
363 2014b; Tréguier et al. 2014). Our protocols followed the most updated guidelines for the use
364 of eDNA for aquatic monitoring (Goldberg et al. 2016), ensuring the consistency of our
365 results. We first validated our method with positive controls and by detecting both native and

366 signal crayfish in sites where they had been previously observed as well as detecting *A. astaci*
367 in a recognised infected river.

368 Only native or invasive crayfish (not both species coexisting) were expected in the
369 Wye catchment, where some populations of *P. leniusculus* are known to be carriers of the
370 plague and have been established for a sufficient amount of time to entirely displace native *A.*
371 *pallipes* from most of the species' historical locations (Dunn et al. 2009; James et al. 2014b),
372 and this was supported by our results. Our multiplex approach successfully identified *A.*
373 *astaci* in the Bachowey stream and *P. leniusculus* in an associated pond less than 10 m from
374 this stream, revealing the presence of infected crayfish further upstream than previously
375 detected (James et al. 2017), despite previous intensive trapping of *P. leniusculus*, which
376 removed 36,000 individuals from the area between 2006 and 2008 (Wye & Usk Foundation
377 2012). We also detected the endangered crayfish *A. pallipes* in spite of its very low
378 abundance in the Sgithwen, made apparent by lack of trapping success, highlighting the
379 sensitivity of the method.

380 In the rivers Medway and Itchen, where invasions date back to the 1970s (NBN
381 2009), both *P. leniusculus* and *A. pallipes* had been previously reported but the crayfish
382 plague status was unknown. We did not find *A. astaci* DNA in any samples from either
383 catchment but found both the native and the invasive species coexisting in at least two
384 sampling sites. This could be explained by the absence of plague, as *A. astaci* is often the
385 main cause of *A. pallipes* population declines (Haddaway et al. 2012). We consistently
386 detected both species over two sampling periods in the Medway, highlighting the
387 reproducibility of the results, which combined with the absence of crayfish plague DNA
388 presence suggests this could be a location where both species' populations are stable (Bubb et
389 al. 2005; Kozubíková et al. 2008). Populations of *A. pallipes* and *P. leniusculus* can coexist
390 for a substantial length of time (c.25 years), as has been observed in other invasive-native

391 crayfish population assemblages (Kozubíková et al. 2008; Peters and Lodge 2013; Schrimpf
392 et al. 2012), providing that there is no introduction of *A. astaci* (Kozubíková et al. 2008;
393 Schrimpf et al. 2012). However, due to competitive exclusion, it is unlikely that populations
394 of both species will coexist indefinitely (Schrimpf et al. 2012; Westman et al. 2002),
395 therefore areas where they overlap should be prioritised for management and control of the
396 invasive species.

397 Detectability was variable among sampling seasons. There were more positive *P.*
398 *leniusculus* field samples from the sampling of Wye sites in October 2016 compared to the
399 samples collected in July 2015 from the same sites, with three and one positive samples
400 respectively. For *A. pallipes*, the only positive field samples for the Welsh sites were from
401 samples collected in October, however eDNA from both *P. leniusculus* and *A. pallipes* was
402 successfully detected in the Medway samples collected in June and July.
403 Seasonal differences could be due to the influence of temperature on eDNA detection rates
404 among aquatic species; with every 1.02 $^{o}$C rise in temperature, species are 1.7 times less
405 likely to be detected, especially if the populations are at very low abundance (Moyer et al.
406 2014), whereas time since DNA release seems to have less effect on detectability at constant
407 temperature (Eichmiller et al. 2016; Moyer et al. 2014). As temperatures in the Wye
408 catchment were around six degrees colder in the stream sites and up to 14 degrees colder in
409 still water bodies in October compared to July, this could explain the differences in detection
410 success among samplings in the Wye catchment (Eichmiller et al. 2016; Moyer et al. 2014).
411 However, temperatures in the Medway were similar to those in the Wye in July suggesting
412 that the differences in detectability between catchments could be due to differences in
413 population size or to local environmental conditions increasing DNA degradation rates in the
414 Wye (Barnes et al. 2014; Dougherty et al. 2016; Jane et al. 2015; Pilliod et al. 2014). In
415 contrast, *A. astaci* sporulation occurs most efficiently at temperatures nearer 20 $^{o}$C, which

could result in more spores being present in the river system in the summer months in comparison to any other time of the year (Wittwer et al. 2018). Released zoospores can only survive up to three days without a host and encysted spores survive up to two weeks in water, particularly during summer months when average temperatures of flowing and enclosed waterbodies are above 18 ºC (Diéguez-Uribeondo et al. 1995; Unestam 1966), meaning it is possible to achieve a relatively real-time picture of *A. astaci* prevalence in eDNA samples (Wittwer et al. 2018). Lower abundance of *A. astaci* spores in colder temperatures could explain lack of detection of *A. astaci* in the October samples at the positive July sites in the Wye catchment (Strand et al. 2014; Wittwer et al. 2018), although detection levels could also have been affected by natural variation in population levels of plague infection (James et al. 2017). Considering this variability, seasonal samplings repeated over at least two years are advisable to reliably map the presence/absence of native and invasive crayfish and determine their infectious status.

In contrast to other essays developed for crayfish detection (Agersnap et al. 2017; Cai et al. 2017; Dougherty et al. 2016; Mauvisseau et al. 2018), our single, closed tube reaction, reduces not only the processing time and number of reactions but also the risk of contamination inherent to carry out a larger number of amplifications. HRM has already proved highly specific and useful for multiple species identification (Naue et al. 2014) and for the management of terrestrial invasive species (Ramón-Laca et al. 2014) but had never been applied to the detection of aquatic invasive species and their impacts using eDNA. Implementation of our multiplex assay provided three-fold biological information (invasive/native/pathogen) on target species', which allows to assess potential contributing factors to native crayfish decline (such as the presence of invasive crayfish and crayfish plague) with greater sensitivity, specificity and efficiency than trapping (Barnes and Turner

2015) or single-species assays, essential to inform effective conservation and management strategies (Darling and Mahon 2011; Kelly et al. 2014).

While most studies have mainly focussed on crayfish eDNA detection in closed systems (Agersnap et al. 2017; Cai et al. 2017; Dougherty et al. 2016; Mauvisseau et al. 2018), our method has also proved useful for monitoring in flowing water bodies. This is important for early detection of invasive crayfish which use rivers and streams as a means for dispersal (Bubb, Thom and Lucas 2004), and particularly for *A. pallipes* whose detection was marginally better using eDNA (7%) than trapping (0%). In terms of sampling effort, eDNA tends to be more time effective than trapping (Smart et al. 2015). However, we failed to detect crayfish in the deep reservoir at Pant-Y-Llyn using eDNA, where trapping had revealed the presence of *P. leniusculus*. Taxonomic groups such as fish and amphibians shed significantly more DNA into the environment compared to invertebrate species, especially those with a hardened exoskeleton such as the crayfish (Thomsen et al. 2012; Tréguier et al. 2014). This reduced release of extracellular DNA can lower the detectability of crayfish, resulting in an increased occurrence of false negatives (Ikeda et al. 2016), particularly when the concentration of DNA is low due to few individuals or large water volumes (Tréguier et al. 2014). The nature of the crayfish exoskeleton combined with the depth of the reservoir prevented samples being taken near the sediment where the crayfish reside could account for observed lack of detection at the Pant-y-Llyn site (Tréguier et al. 2014). Collection of sediment samples in addition to water could improve levels of detection of target species, because DNA from sediment can last longer and be more concentrated than in water (Turner et al. 2015).

Conservation efforts rely on efficient, standardised methods for collecting biological data, which advance beyond the limitations of traditional sampling methods (Thomsen and Willerslev, 2015). Ecosystem management and conservation strategies strive to protect

biodiversity through preventing invasions from novel species (thus the need for early detection) and effectively monitoring rare native species to preserve hotspots and ark sites (Lodge et al. 2012). Environmental DNA has been directly used as a conservation tool to survey both invasive (e.g.Takahara et al. 2013; Tréguier et al. 2014) and endangered native species (e.g. Olson et al. 2012; Sigsgaard et al. 2015) and we have shown how an eDNA-based qPCR-HRM multiplex approach can identify invasive hosts and their pathogens as well as refugia for the native species. This was particularly important to identify areas of coexistence between aquatic native and invasive crayfish (e.g. at the early stages of invasion or where crayfish plague is absent) (Schrimpf et al. 2012), which could be prioritised for long-term conservation plans.

Incorporating this tool to monitoring programmes for conservation significantly reduces the costs of sample processing compared to species' targeted methods. Our method can ultimately help in the early detection and prevention of dispersal of invasive hosts and pathogens in threatened freshwater ecosystems, as well as in determining suitable locations for the potential reintroduction of the native species to historic habitats. As genomic technology advances, environmental DNA assays should continue to provide additional information, including more accurate data on species abundance and biomass in both lotic and lentic systems (Bohmann et al. 2014; Rees et al. 2014) as well as development of additional multiplexes to simultaneously detect numerous target species of conservation interest.

## ACKNOWLEDGEMENTS

This research was funded by the Welsh Government and Higher Education Funding Council for Wales (HEFCW) through the Sêr Cymru National Research Network for Low Carbon Energy and Environment (AQUAWALES; NRN-LCEE) and by the Environment Agency

UK. We thank: Jennifer Nightingale, Oliver Brown and Adam Petrusek for crayfish/DNA samples, Stephen Marsh-Smith, Louis MacDonald-Ames & Hayden Probert, for logistics and information on crayfish trapping; Tony Rees and members of Merthyr Tydfil Angling Club water sample collection; Hampshire and Isle of Wight Wildlife Trust (Dr. Ben Rushbrook) and Environment Agency (Kerry Walsh, Emma McSwan, Kathy Friend) for assistance with sample collection and expertise in the Itchen and Medway and also Carlos Garcia de Leaniz for valuable advice on experimental design and comments to the final manuscript.

## Author contributions & competing interests

SC & CVR designed the study; CVR & TUW performed the analyses; JC & JJ contributed samples and information; SC & CVR wrote the paper with the help of all the authors. Authors declare that they have no competing interests.

## Data Accessibility

All data is currently included in the supplementary material and will be stored in Dryad upon acceptance if requested.

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771

772 **Table 1**. Location and environmental data for eDNA sampling sites in the River Wye for July 2015 and October 2016 (in italics); River Taff
773 (May 2015); River Medway July 2016 and June 2017 (in italics) and the River Itchen (October 2017), including waterbody type, GPS
774 coordinates, shade cover (0-3), temperature (ºC), flow rate (m/s) and total number of samples collected per site minus negative controls (three
775 samples in duplicate (6) or triplicate (9)).

| Date | Waterbody | Crayfish Status | Site No. | Waterbody Type | GPS Coordinates | Shade Cover | Temperature (ºC ) | Flow Rate (m/s) | No. Samples |
|---|---|---|---|---|---|---|---|---|---|
| 10/07/2015 | Sgithwen | Native | 1 | Stream | SO 11152 41419 | 3 | 15 | N/A | 9 |
| *14/10/2016* | *Sgithwen* | *Native* | *1* | *Stream* | *SO 11152 41419* | *2* | *8.5* | *0.2* | *9* |
| 10/07/2015 | Sgithwen | Native | 2 | Stream | SO 10819 41423 | 3 | 14 | N/A | 3 |
| *14/10/2016* | *Sgithwen* | *Native* | *2* | *Stream* | *SO 10819 41423* | *2* | *9* | *0.2* | *9* |
| 10/07/2015 | Bachowey | Signal | 3A | Stream | SO 10623 42814 | 2 | 14 | N/A | 3 |
| 10/07/2015 | Bachowey | Signal | 3B | Stream | SO 13821 45723 | 1 | 15 | N/A | 3 |
| *14/10/2016* | *Bachowey* | *Signal* | *3B* | *Stream* | *SO 13821 45723* | *0* | *9* | *0.3* | *9* |
| 10/07/2015 | Bachowey | Signal | 3C | Pond | SO 18504 47170 | 0 | 23 | N/A | 3 |
| *14/10/2016* | *Bachowey* | *Signal* | *3C* | *Pond* | *SO 18504 47170* | *0* | *8.5* | *0.5* | *9* |
| 10/07/2015 | Bachowey | Signal | 4 | Stream | SO 18562 47118 | 1 | 15 | N/A | 9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *14/10/2016* | *Bachowey* | *Signal* | *4* | *Stream* | *SO 18562 47118* | *1* | *8* | *0.2* | *9* |
| 15/07/2015 | Edw | Unknown | 5A | Stream | SO 08471 47124 | 2 | 16 | N/A | 3 |
| 15/07/2015 | Edw | Unknown | 5B | Stream | SO 11226 48715 | 2 | 15 | N/A | 3 |
| 15/07/2015 | Edw | Unknown | 5C | Stream | SO 12409 52102 | 2 | 16 | N/A | 3 |
| 15/07/2015 | Duhonw | Signal | 6A | Stream | SO 03831 48780 | 2 | 15 | N/A | 3 |
| 15/07/2015 | Duhonw | Signal | 6B | Stream | SO 02837 47179 | 3 | 14 | N/A | 3 |
| 15/07/2015 | Duhonw | Signal | 6C | Pond | SO 03891 46490 | 0 | 23 | N/A | 3 |
| 01/05/2016 | Taff | Unknown | T1 | Pond | SO 07192 08525 | 0 | 10 | N/A | 6 |
| 01/05/2016 | Taff | Unknown | T2 | Pond | SO 07195 08318 | 0 | 12 | N/A | 6 |
| 01/05/2016 | Taff | Unknown | T3 | Lake | SO 03963 07262 | 1 | 13 | N/A | 6 |
| 01/05/2016 | Taff | Unknown | T4 | Stream | SO 03719 07681 | 0 | 11 | 0.3 | 6 |
| 01/05/2016 | Taff | Unknown | T5 | Stream | SO 03756 07480 | 2 | 10 | 0.3 | 6 |
| 01/05/2016 | Taff | Unknown | T6 | Lake | SO 00849 11346 | 0 | 13 | N/A | 6 |
| 01/05/2016 | Taff | Unknown | T6 | Stream | SO 01560 10665 | 2 | 11 | 0.2 | 6 |
| | | | | | | | | | |
| 27/07/16 | Medway | Signal | 1 | River | TQ 59089 46489 | 0 | 17 | 0.2 | 6 |
| *29/06/17* | *Medway* | *Signal* | *1* | *River* | *TQ 59089* | *0* | *15* | *0.2* | *6* |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | *46489* | | | | |
| 27/07/16 | Medway | Signal | 2 | Stream | TQ 67472 48254 | 0 | 15 | 0.3 | 6 |
| 27/07/16 | Medway | Unknown | 3 | Pond | TQ 60810 51347 | 2 | 17 | N/A | 6 |
| *29/06/17* | *Medway* | *Unknown* | *3* | *Pond* | *TQ 60810 51347* | *3* | *19* | *N/A* | *6* |
| 27/07/16 | Medway | Signal | 4 | River | TQ 68987 49924 | 1 | 18 | 0.2 | 6 |
| 27/07/16 | Medway | Signal | 5 | River | TQ 72866 48687 | 1 | 16 | 0.3 | 6 |
| *29/06/17* | *Medway* | *Signal* | *5* | *River* | *TQ 72866 48687* | *1* | *18* | *0.3* | *6* |
| 27/07/16 | Medway | Signal | 6 | Stream | TQ 77297 46511 | 3 | 14 | 0.1 | 6 |
| 27/07/16 | Medway | Signal | 7 | Stream | TQ 72843 45680 | 1 | 13 | 0.2 | 6 |
| *29/06/17* | *Medway* | *Signal* | *7* | *Stream* | *TQ 72843 45680* | *1* | *15* | *0.2* | *6* |
| 27/07/16 | Medway | Unknown | 8 | River | TQ 70880 53290 | 1 | 15 | 0.2 | 6 |
| 27/07/16 | Medway | Unknown | 9 | River | TQ 73478 53564 | 0 | 16 | 0.4 | 6 |
| *29/06/17* | *Medway* | *Unknown* | *9* | *River* | *TQ 73478 53564* | *0* | *18* | *0.4* | *6* |
| 27/07/16 | Medway | Unknown | 10 | River | TQ 75665 55630 | 0 | 17 | 0.3 | 6 |
| *29/06/17* | *Medway* | *Unknown* | *10* | *River* | *TQ 75665 55630* | *0* | *19* | *0.3* | *6* |
| 27/07/16 | Medway | Unknown | 11 | Lake | TQ 70192 59812 | 2 | 19 | N/A | 6 |
| *29/06/17* | *Medway* | *Unknown* | *11* | *Lake* | *TQ 70192* | *3* | *20* | *N/A* | *6* |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 12.10.17 | Itchen | Native | 1 | Stream | SU 56614 36671 | 2 | 12 | 0.3 | 6 |
| 12.10.17 | Itchen | Native | 2 | Stream | SU 56333 35363 | 2 | 13 | 0.1 | 6 |
| 12.10.17 | Itchen | Native | 3 | Stream | SU 56369 34569 | 0 | 12 | 0.1 | 6 |
| 12.10.17 | Itchen | Native | 4 | River | SU 56853 32348 | 1 | 12 | 0.5 | 6 |
| 12.10.17 | Itchen | Native | 5 | River | SU 56831 31976 | 1 | 12 | 0.3 | 6 |
| 12.10.17 | Itchen | Native | 6 | Stream | SU 57986 29401 | 0 | 12 | 2.0 | 6 |
| 12.10.17 | Itchen | Native | 7 | Stream | SU 57253 31065 | 2 | 13 | 0.5 | 6 |
| 12.10.17 | Itchen | Native | 8 | Stream | SU 57379 31646 | 0 | 12 | 0.3 | 6 |
| 12.10.17 | Itchen | Native | 9 | Stream | SU 57242 32325 | 1 | 13 | 1.5 | 6 |
| 12.10.17 | Itchen | Native | 10 | River | SU 56875 31912 | 3 | 13 | 1.0 | 6 |
| 12.10.17 | Itchen | Native | 11 | River | SU 56415 31826 | 2 | 12 | 0.1 | 6 |
| 12.10.17 | Itchen | Signal | 12 | River | SU 60133 32401 | 2 | 12 | 0.1 | 6 |
| 12.10.17 | Itchen | Signal | 13 | River | SU 58473 33218 | 1 | 12 | 3.0 | 6 |
| 12.10.17 | Itchen | Signal | 14 | River | SU 58490 33129 | 3 | 12 | 0.5 | 6 |
| 12.10.17 | Itchen | Signal | 15 | River | SU 58402 33025 | 2 | 12 | 0.1 | 6 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 12.10.17 | Itchen | Native | 16 | River | SU 57413 32600 | 3 | 12 | 0.5 | 6 | 776 |
| 12.10.17 | Itchen | Signal | 17 | River | SU 53545 32705 | 3 | 12 | 0.5 | 6 | |
| 12.10.17 | Itchen | Signal | 18 | River | SU 51113 32501 | 3 | 12 | 0.3 | 6 | |

777 **Table 2.** Location of crayfish traps in corresponding waterbodies in the Wye catchment and number of crayfish caught per trap.

| **Waterbody** | **Temp (ºC)** | **Flow Rate (m/s)** | **pH** | **Trap depth (m)** | **No. Traps** | **No. Crayfish Caught** | **No. Traps Containing Crayfish** | **Trap Coordinates** | **No. Samples** | ***Pacifastacus leniusculus* DNA detected?** |
|---|---|---|---|---|---|---|---|---|---|---|
| Sgithwen | 12 | 1.5 | 7.40 | 1 | 2 | 0 | 0 | SO 11190 41410<br>SO 10750 42740 | 0 | N/A |
| Bachowey Stream | 12 | 0.4 | 7.60 | 0.75 | 11 | 3 | 2 | SO 10750 42740<br>SO 13800 45700<br>SO 18560 47130<br>SO 18477 47077<br>SO 17150 46130<br>SO 18514 47107 | 6 | No |
| Bachowey Pond | 18 | N/A | 7.40 | 1 | 4 | 0 | 0 | SO 18540 47180 | 0 | N/A |
| Edw River | 13 | 0.3 | 7.50 | 1 | 3 | 0 | 0 | SO 12407 52105<br>SO 11210 48690<br>SO 08473 47123 | 0 | N/A |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Duhonw River | 11 | 0.6 | 7.40 | 1 | 2 | 0 | 0 | SO 03776 48781<br>SO 02774 47102 | 0 | N/A |
| Pant-y-Llyn Reservoir | 18 | N/A | 7.40 | >1 | 3 | 8 | 3 | SO 18498 47083 | 9 | Yes |

778 **Table 3.** Summary of average values from qPCR outputs for both *Pacifastacus leniusculus* and *Austropotamobius* pallipes. Average melt
779 temperature (°C; Avg. tm); Average melt peak height (Avg. peak height); Average start melt temperature (°C; Avg. start melt); Average end melt
780 temperature (°C; Avg. end melt) of resultant qPCR products with standard deviation. Values were obtained for each individual over at least three
781 separate runs, each consisting of three replicates and negative control blanks.

| **Species/Master Mix** | **Sample size (N)** | **Avg. tm (°C) (±SD)** | **Avg. peak height (±SD)** | **Avg. start melt (°C) (±SD)** | **Avg. end melt (°C) (±SD)** |
|---|---|---|---|---|---|
| *Pacifastacus leniusculus* / SYBR® Green | 15 | 72.7 (0.2) | 376.3 (40.8) | 69.5 (0.4) | 75.5 (0.3) |
| *Austropotamobius pallipes* / SYBR® Green | 12 | 73.6 (0.2) | 382.7 (30.7) | 70.4 (0.3) | 76.7 (0.3) |
| *Pacifastacus leniusculus* / SsoFast™ EvaGreen® | 15 | 73.9 (0.2) | 397.6 (36.4) | 71.1 (0.2) | 76.6 (0.2) |

| | | | | | |
|---|---|---|---|---|---|
| *Austropotamobius pallipes* / SsoFast™ EvaGreen® | 12 | 74.8 (0.2) | 449.1 (21.6) | 71.8 (0.3) | 77.2 (0.2) |

782 **Table 4.** Melt data from SsoFast™ EvaGreen® eDNA qPCR amplifications for the Taff
783 catchment.

| Mastermix | Catchment | Sample ID | Melt Temperature (°C) |
|---|---|---|---|
| SsoFast™ EvaGreen® | Taff | 5B | 73.80 |
| SsoFast™ EvaGreen® | Taff | 5B | 73.80 |
| SsoFast™ EvaGreen® | Taff | 5B | 72.80 |
| SsoFast™ EvaGreen® | Taff | 5B | 74.00 |
| SsoFast™ EvaGreen® | Taff | 5C | 73.80 |
| SsoFast™ EvaGreen® | Taff | 5C | 73.80 |
| SsoFast™ EvaGreen® | Taff | 5C | 72.90 |
| SsoFast™ EvaGreen® | Taff | 5C | 73.60 |
| SsoFast™ EvaGreen® | Taff | 5D | 74.00 |
| SsoFast™ EvaGreen® | Taff | 5D | 73.80 |
| SsoFast™ EvaGreen® | Taff | 5D | 73.70 |
| SsoFast™ EvaGreen® | Taff | PC_SC | 73.70 |
| SsoFast™ EvaGreen® | Taff | PC_SC | 73.70 |
| SsoFast™ EvaGreen® | Taff | PC_SC | 73.70 |
| SsoFast™ EvaGreen® | Taff | PC_NC | 74.70 |
| SsoFast™ EvaGreen® | Taff | PC_NC | 74.70 |
| SsoFast™ EvaGreen® | Taff | PC_NC | 74.70 |
| SsoFast™ EvaGreen® | Taff | MB | None |
| SsoFast™ EvaGreen® | Taff | MB | None |
| SsoFast™ EvaGreen® | Taff | MB | None |
| SsoFast™ EvaGreen® | Taff | MB | None |
| SsoFast™ | Taff | MB | None |

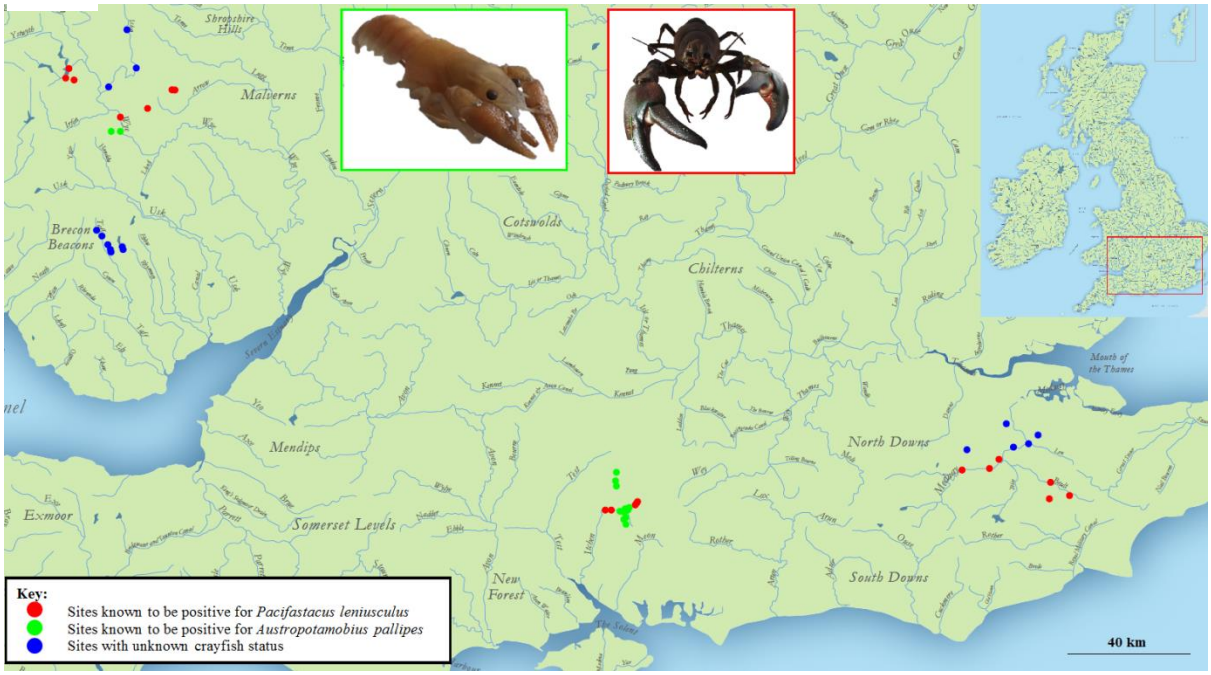
| | | | |
|---|---|---|---|
| EvaGreen® SsoFast™ EvaGreen® | Taff | MB | None |

Sample ID: # Taff catchment sample with corresponding subsample letter, PC_SC Signal crayfish positive DNA control, PC_NC Native crayfish positive DNA control, MB Amplification negative control

811 **Figure 1a.** eDNA sampling sites for England (Medway and Itchen) and Wales (Wye and
812 Taff) in tributaries with known presence of *Pacifastacus leniusculus* individuals (red circle),
813 *Austropotamobius pallipes* (green circle) or without information regarding crayfish status
814 (blue circle). Each point represents a locality where between three and nine water samples
815 were collected. (*Austropotamobius pallipes* photograph ©Chloe Robinson; *Pacifastacus*
816 *leniusculus* photograph ©Rhidian Thomas).

817

818 **A**

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821 **Figure 1b**. Location of the rivers Wye and Taff eDNA sampling sites in Wales. Wye sites 1
822 and 2 (Sgithwen Brook) were confirmed for crayfish species *Austropotamobius pallipes*; sites
823 3 (Bachowey), 4 (Bachowey) and 6 (Duhonw) were confirmed for crayfish species
824 *Pacifastacus leniusculus* and site 5 (Edw) had unknown crayfish status. Taff sites T1 to T7 all
825 had unknown crayfish presence status. Each point corresponds to between three and nine
826 water samples collected

827 B

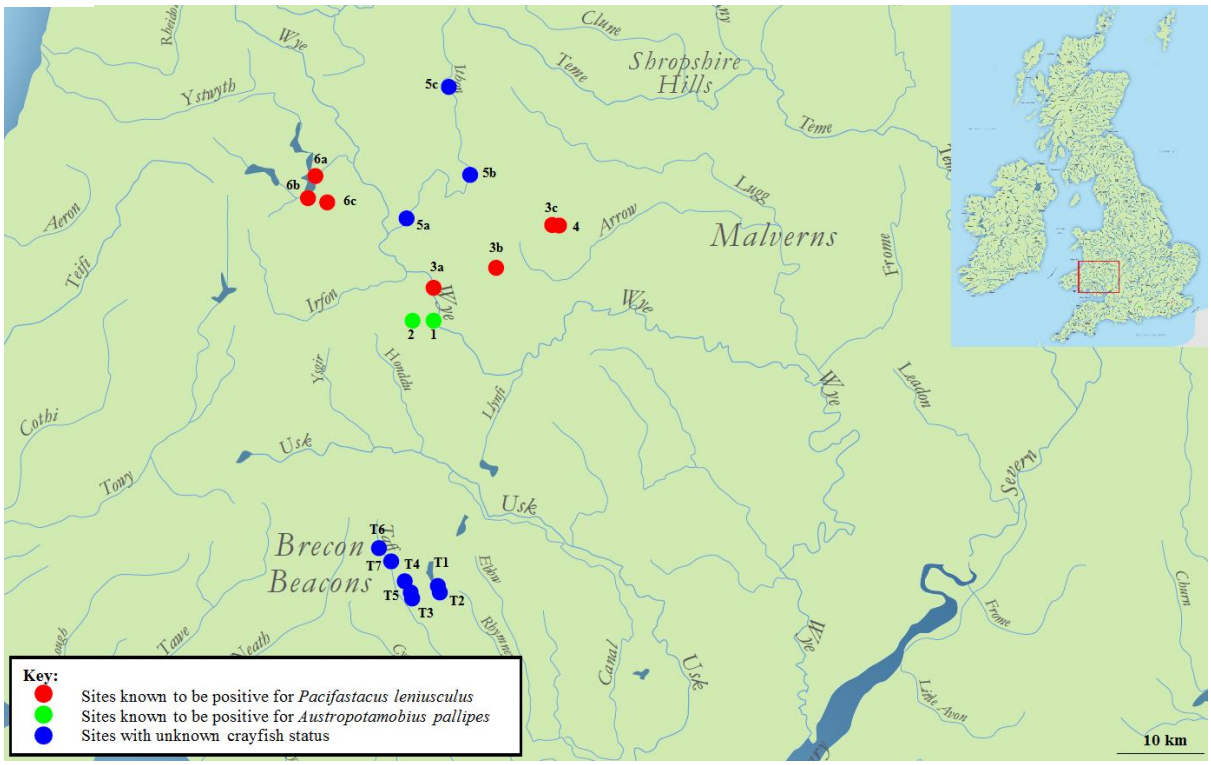


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**Figure 1c**. Locations of the rivers Itchen and Medway eDNA sampling sites. In the Itchen, there were 18 sites in total (I1 to I18); I1 – I11 classified as positive for *Austropotamobius pallipes* presence and I12 – 18 classified as positive for *Pacifastacus leniusculus* presence. In the Medway, there were 11 sites in total (M1 to M11); M1, M2 – M6 were classified as positive for *Pacifastacus leniusculus* presence whereas M3, M8 – M11 have an unknown crayfish species status. Each point corresponds to six water samples collected.

C

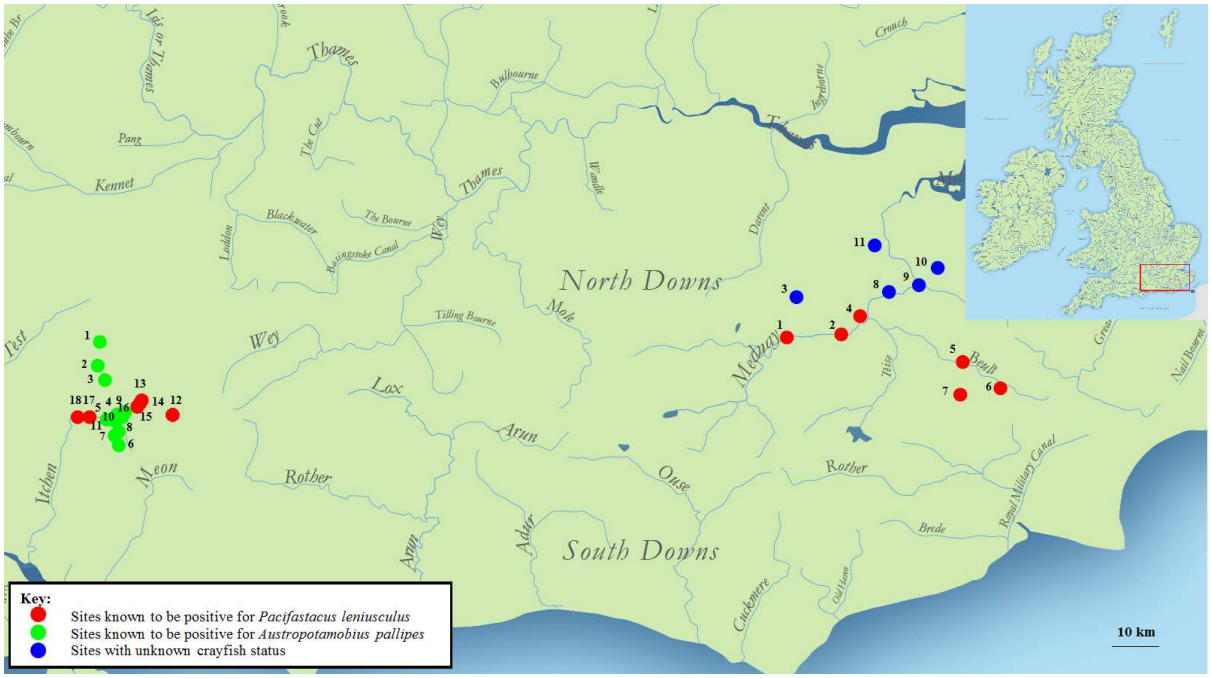


**Figure 2.** qPCR product melt peak output for multiplex amplification of DNA using optimised HOT FIREPol® EvaGreen® from three different *Pacifastacus leniusculus* individuals and *Aphanomyces astaci* DNA in the same qPCR reaction (Pool 1-3), displaying the diagnostic double melt peaks at 75.9 ± 0.2 °C for *Pacifastacus leniusculus* and 82.9 °C for

*Aphanomyces astaci*. Also displayed, is the qPCR melt profiles for mixed proportions of *Pacifastacus leniusculus* and *Austropotamobius pallipes* DNA in the same qPCR reaction, showing the diagnostic melt shape difference between the three types of amplification (circled) and positive controls. Mix 1 = 90:10 *Pacifastacus leniusculus*: *Austropotamobius pallipes*, Mix 2 = 50:50 *Pacifastacus leniusculus*: *Austropotamobius pallipes*, PL = *Pacifastacus leniusculus* and AP = *Austropotamobius pallipes.*

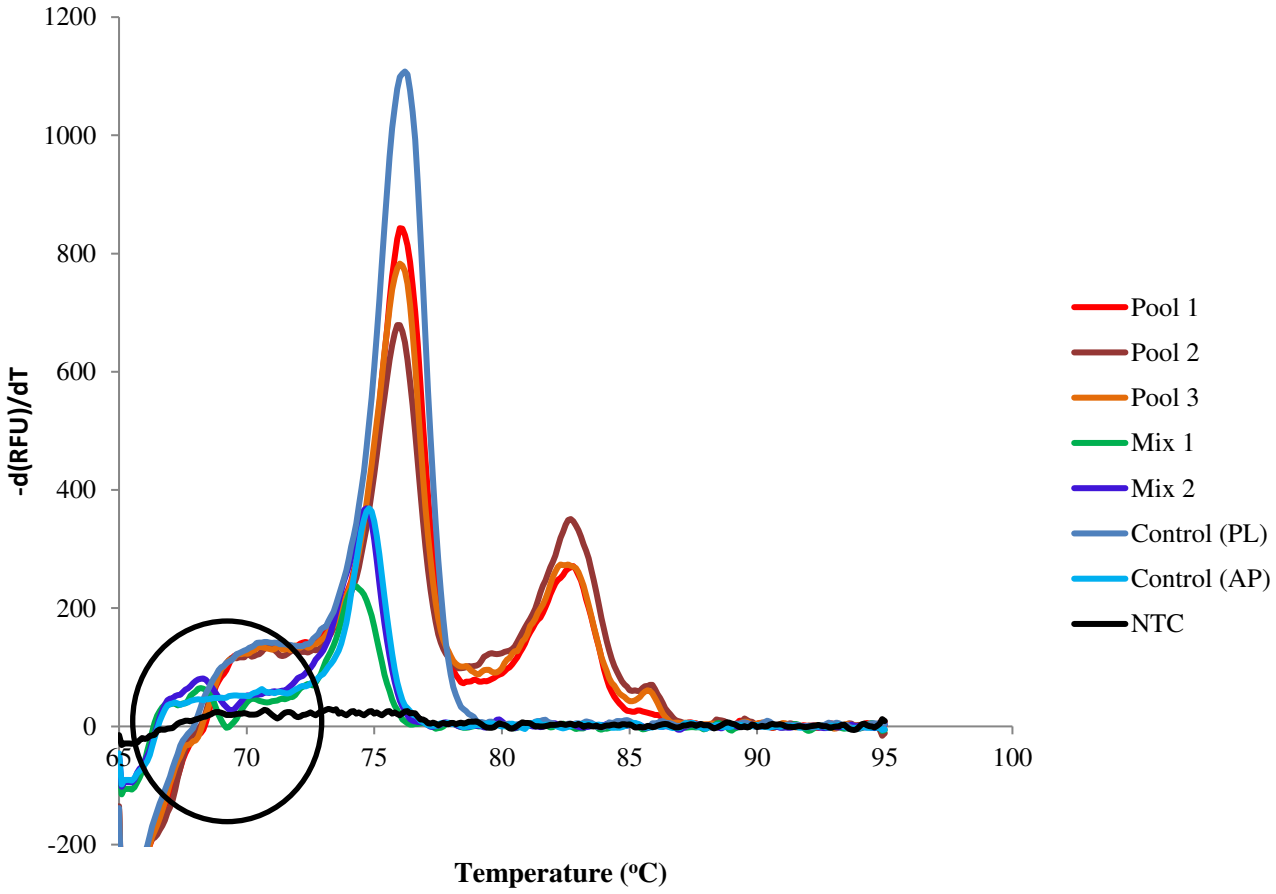


**Figure 3**. Melt peak profile for HOT FIREPol® EvaGreen® multiplex amplification of samples 5B, 5C and 5D from site 5 in the Taff catchment. The two largest sets of peaks correspond to positive control tissue for both *Pacifastacus leniusculus* (73.7 °C) and

876 *Austropotamobius pallipes* (74.7 ºC) and subsequent peaks represent eDNA field sample melt
877 peaks for both *Pacifastacus leniusculus* and *Austropotamobius pallipes*. Non-template had no
878 melt profile (flat line).

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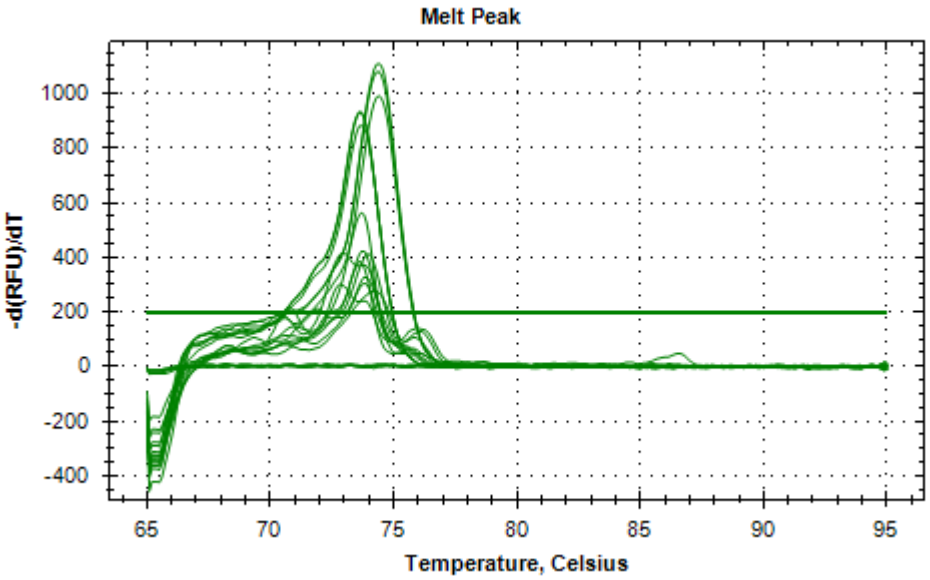


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**Figure 4**. Melt peak profile for SsoFast™ EvaGreen® eDNA qPCR amplifications of positive amplifications for both *Pacifastacus leniusculus* and *Austropotamobius pallipes* in the same site. The three largest sets of peaks correspond to positive control tissue (one sample in triplicate) for both *Pacifastacus leniusculus* (74.9 ºC), *Austropotamobius pallipes* (75.9 ºC) and *Aphanomyces astaci* (82.9 ºC). Subsequent peaks represent eDNA field sample melt peaks from nine samples (in triplicate) for both native *Austropotamobius pallipes* and invasive *Pacifastacus leniusculus*, with absence of any melt peak for *Aphanomyces astaci* in field samples. Non-template control has no melt profile (flat line).