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Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using eDNA

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Running headline: Detection of non-native fishes using eDNA

Abstract

This paper presents the first phase in the development and validation of a simple and reliable environmental DNA (eDNA) method using conventional PCR to detect four species of non-native freshwater fish (pumpkinseed *Lepomis gibbosus*, sunbleak *Leucaspius delineatus*, fathead minnow *Pimephales promelas* and topmouth gudgeon *Pseudorasbora parva*). The efficacy of the approach was demonstrated in indoor (44-L tank) trials in which all four species were detected within 24 hours. Validation was through two field trials, in which *L. gibbosus* was detected 6–12 hours after its introduction into outdoor experimental ponds, and *P. parva* was successfully detected in disused fish rearing ponds where the species was known to exist. Thus, the filtration of small (30 mL) volumes of pond water was sufficient to capture fish eDNA and the approach emphasised the importance of taking multiple water samples of sufficient spatial coverage for detecting species of random or patchy distribution.

Key words: environmental DNA, *Pseudorasbora parva, Leucaspius delineatus, Lepomis gibbosus, Pimephales promelas*

INTRODUCTION

The most economical and effective means of preventing biological invasions is early detection and eradication (Hulme, 2009; Gozlan *et al.*, 2010). In recent years, a very promising molecular approach for early detection of non-native species has been developed (Ficetola *et al*., 2008), based on analysis of DNA shed by an organism into the environment (eDNA), e.g. via urine, faeces, mucus and epidermal cells (Bohmann *et al.*, 2014). Recent studies have shown that eDNA approaches are applicable for detecting a range of aquatic taxa (Thomsen *et al.*, 2012a), including amphibians (e.g. Dejean *et al.*, 2012; Pilliod *et al.*, 2014; Rees *et al.*, 2014a; Biggs *et al.*, 2015), mammals (e.g. Foote *et al.*, 2012), invertebrates (e.g. Goldberg *et al.*, 2013; Deiner & Altermatt, 2014), and fishes (e.g. Keskin, 2014; Gustavson *et al.*, 2015). Indeed, eDNA methods are particularly applicable to fishes, such as for detecting species' range expansions (e.g. Jerde *et al.*, 2011, 2013), establishing species distributions (e.g. Takahara *et al.*, 2013; Keskin, 2014) and inventories for fish assemblages in rivers (e.g. Minamoto *et al.*, 2012) and inshore marine waters (Thomsen *et al.*, 2012b).

Surveys that employ eDNA techniques can complement conventional sampling methods, usually proving to be more effective for detecting elusive and/or rare species (e.g. Janosik & Johnston, 2015; Sigsgaard *et al.*, 2015). In some cases, such as surveys of invasive American bullfrogs *Lithobates catesbeianus* (Shaw 1802) and native great crested newts *Triturus cristatus* (Laurenti 1768), detection rates in ponds were higher using eDNA methods (Dejean

62 *et al*., 2012; Biggs *et al*., 2015), and the financial cost was approximately 10× cheaper than
63 with conventional methods (Biggs *et al.*, 2015). Therefore, eDNA analysis shows great
64 potential as a relatively rapid means of surveying a large number of water bodies with no
65 adverse effect on the environment, with fewer restrictions caused by weather conditions, and
66 at relatively low financial cost (Rees *et al.*, 2014b). The information obtained from such
67 surveys can be applied to facilitate effective management of non-native species, for example
68 by detecting newly-arrived invaders and enabling a rapid response (Vander Zanden *et al*.,
69 2010); by accurately mapping the distributions of established species (Dejean *et al*., 2012;
70 Jerde *et al*., 2013); and by providing a means to assess the success of eradication programmes
71 (Rout *et al*., 2009).

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74 The aim of the present study was to develop and validate a simple, relatively low-cost
75 eDNA protocol for surveying four species of freshwater fish that are non-native to the UK, in
76 order to inform management decisions. Topmouth gudgeon *Pseudorasbora parva*
77 (Temminck & Schlegel 1846), native to Asia, was accidentally introduced to the UK in the
78 mid-1980s as a contaminant of ornamental fish, and subsequently spread to form scattered
79 populations throughout England and Wales (Gozlan *et al*., 2002). As an invasive species
80 known to have a detrimental effect on native fish communities, all 23 known populations are
81 targeted for eradication from the UK by 2017 (Britton *et al*., 2010a; GBNNSS 2015).
82 Sunbleak *Leucaspius delineatus* (Heckel 1843), native to continental Europe, is also
83 considered invasive in the UK, and has spread to a number of locations in southern England
84 following its introduction in the mid-1980s with ornamental fish (Zięba *et al*., 2010a). The
85 other two species, pumpkinseed *Lepomis gibbosus* (L. 1758) and fathead minnow *Pimephales*

promelas Rafinesque 1820, both native to North America, have established populations in the UK without yet becoming invasive. However, they are predicted to become invasive in the UK under warmer climatic conditions (Britton *et al.*, 2010b). *Lepomis gibbosus* was first introduced to the UK as an ornamental fish during the 1890s or early 1900s (Lever, 1977), and now occurs at a number of locations in southern England (Villeneuve *et al.*, 2005). No populations of *P. promelas* are currently known from the UK, but this species was previously established in two adjacent ponds in northern England, probably arriving as a contaminant of ornamental fish in 1996 and persisting until eradication in 2010 (Zięba *et al*., 2010a).

The specific objectives were to: 1) develop specific primers for the four fish species; 2) test the primers and sampling methodology in aquarium trials, using these to determine time between the introduction of fish and the detection of eDNA, for a known fish biomass; 3) test the methodology in experimental pond trials, again to determine time between introduction of fish and the detection of eDNA, for a known biomass of one of the species (*L. gibbosus*); and 4) conduct a field survey to validate the protocol for one of the species (*P. parva*) in small ponds where the species is known to be present.

MATERIALS AND METHODS

Specimens of three fish species were sourced from established non-native populations in southern England: *L. gibbosus* and *L. delineatus* from a commercial angling venue in East Sussex, England (51·018333° N; 0·013056° E), and *P. parva* from ponds of a decommissioned ornamental fish farm in Hampshire, England (51·000556° N; 1·452778° W). Wild populations of *P. promelas* in the UK were not available, the only known population having been eradicated three years prior to start of the present study (Britton *et al*., 2011), so laboratory-reared specimens were obtained from a captive source (AstraZeneca Environmental Laboratory, Brixham, UK).

DEVELOPMENT OF SPECIFIC PRIMERS

Specific primers for the four species (Table I), targeting the mitochondrial gene encoding cytochrome *c* oxidase subunit 1 (COI), were designed using the NCBI Primer-BLAST software (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), using sequences available from an open source database (GenBank, http://www.ncbi.nlm.nih.gov/genbank/). To test the efficacy and specificity of the primers, tissue samples were taken from the dorsal muscle of each fish species and the mtDNA was extracted using a tissue extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). Amplified samples were purified (Nucleospin Gel and PCR Clean-up, Machery-Nagel, Düren, Germany) and sequenced by a commercial service (Source Bioscience, Cambridge, UK). The sequences have been deposited in Genbank with the following accession numbers: *L. gibbosus* KR092382; *L. delineatus* KR092383; *P. promelas* KR092384; *P. parva* KR092385.

The PCRs were performed in 20 μL reaction mixtures (2 μL of DNA template, 0·5 μM of each primer, 10 μL (= 50 units) HotStar Taq Plus DNA polymerase (Qiagen Fast Cycling PCR Kit) and 2 μL CoralLoad Fast Cycling Dye (Qiagen)). Tests of temperature gradients at the annealing stage indicated that the same optimum PCR programme could be used for all four species. The optimal temperature for detection of both *L. gibbosus* and *P. promelas* was 61·0° C, with the other two species showing equally distinct bands across the temperature range of 58–62° C. The cycling conditions were 95° C for 5 min, followed by 32 cycles of 96° C for 5 sec, 61° C for 5 sec and 68° C for 12 sec, with a final extension at 72° C for 1 min. PCR products were visualised using electrophoresis on 2% agarose gel, stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK).

Sensitivity of the primers was tested by measuring the quantity of DNA extracted from the dorsal muscle tissue using a Nanodrop ND-1000 spectrophotometer (Nanodrop Instruments, Wilmington, DE, USA), and by producing a dilution series from 0·03 ng μL^{-1} to $1{\cdot}8 \times 10^{-6}$ ng μL^{-1}. Three replicate dilution series were conducted for each species.

Primers were designed to amplify relatively long sections of DNA, targeting regions of 251–350 base pairs (Table I) to improve specificity. In addition to *in silico* tests for primer specificity using the Primer Blast software, all four primer pairs were tested against tissue samples from all four species in this study, with no false positives occurring from cross-species amplification.

AQUARIUM TRIALS

The aquarium trials were conducted in 44-L tanks of de-fluorinated tap water, with no flow-through, within an indoor facility at the Cefas Lowestoft Laboratory. The water in these tanks was maintained at a similar temperature to the outside holding facilities used for the fish prior to the experiment, and was exposed to a natural photo-period. Trials were conducted in January 2014 (*L. delineatus* and *L. gibbosus*) and March 2014 (*P. parva* and *P. promelas*). Water temperature measurements were recorded every 10 min using Tinytag Aquatic 2 temperature loggers (Gemini Data Loggers UK Ltd, Chichester, UK). Mean, minimum and maximum (mean; min–max) temperatures (° C) over the five-day experiment were as follows: *P. parva* (6·8°C; 6·5–9·9), *P. promelas* (6·3°C; 5·9–9·0), *L. delineatus* (2·1°C; 1·9–2·3), *L. gibbosus* (2·3°C; 2·2–2·5).

For each species, tanks were stocked with one, five or ten fish to achieve a range of fish biomass, with three replicate tanks for each biomass (Table II). The exception was for one replicate of the high *L. gibbosus* biomass, where a single large specimen of equivalent biomass was used in lieu of smaller specimens due to limited fish availability. Fish were not fed for the duration of the experiment. On completion of the experiment, each fish was measured for total length (L_T) and mass (Table II).

Water samples (1 L) were collected by submerging a sterilized plastic bottle with a gloved hand into the aquarium. Samples were collected from each tank before the addition of the fish

(day 0), and at 24-h intervals following stocking, for five days. Immediately following sample collection, water samples were filtered through a 0·4 μm pore size polycarbonate filter of diameter 47 mm (Isopore, EMD Millipore, Darmstadt, Germany) using a vacuum pump (EMD Millipore). The filter was immediately frozen at –80° C. At six points during filtration, de-ionised water was run through the filtration system and filtered as above, with these samples analysed to detect any potential cross-contamination. Within six months from initial sampling, the DNA was extracted using a PowerWater DNA Isolation Kit (MO BIO, Carlsbad, CA, USA). PCR amplification was performed using the reaction mixtures and cycling conditions described previously, with three replicates for each sample, and including both a negative (de-ionised water) and positive (DNA standard) control. Filtrations, extractions and PCRs were each conducted in separate laboratories to reduce risk of contamination.

EXPERIMENTAL POND TRIAL

A field trial was conducted in six artificial outdoor ponds constructed previously for experimental studies (Zięba *et al.*, 2010b; Fobert *et al.*, 2011) on a fishery site in East Sussex, UK (51·018333° N; 0·013056° E). Each pond was 25 m^2 (5 m × 5 m), configured with a 1 m wide, 0·2–0·5 m deep shelf on one side, with the remainder being ≈ 1·2 m deep (Zięba *et al.*, 2010b), with an approximate volume of 25750 L. Each pond was fitted with a recirculation system (a maximum rate of 2400 L h^{-1}) whereby the water was pumped into a 227-L cistern containing Canterbury spar gravel through which it filtered before returning to the pond via an overflow pipe. The ponds were enclosed within a netting cage to prevent mammalian or avian predation during the experiment. Pond temperatures were measured every 10 min using

Gemini Tinytag Aquatic 2 data loggers. Mean temperature during the 48 h of the experiment was 21·2° C (min–max = 18·2–24·6).

Prior to commencing, the ponds were drained to ensure the absence of fish, and then left to re-fill with rain-water. A semi-natural and representative fish community was then created by stocking three species of native fish (15 roach *Rutilus rutilus* (L. 1758), nine rudd *Scardinius erythrophthalmus* (L. 1758), and 10 tench *Tinca tinca* (L. 1758)) into the ponds three months prior to *L. gibbosus* introduction. The mean, minimum and maximum (mean ± SE; min–max) biomasses of native fish in each pond, calculated from fish L_T at time of stocking using published length-weight equations (Britton & Shepherd, 2005; Verreycken *et al.*, 2011), were: *R. rutilus* (46·2 ±0·6 g, 44·1–48·5); *S. erythrophthalmus* (40·1 ±2·3 g, 36·3–49·4); *T. tinca* (56·1 ±5·3 g, 38·2–71·2).

Lepomis gibbosus were captured using electro-fishing and minnow traps from the fishery's commercial angling ponds, and 30 specimens were stocked into three of the six experimental ponds in June 2014. The mean, minimum and maximum (mean ±SE, min–max) values of L_T for *L. gibbosus* stocked into the three ponds were: Pond 1 (55·4 ±3·8 mm, 42–131); Pond 2 (57·8 ±4·0 mm, 44–123); Pond 3 (58·3 ±4·4 mm, 43–136). Fish biomass in each experimental pond was calculated from L_T using a length-weight equation derived from *L. gibbosus* measurements from the same commercial angling lakes (Villeneuve *et al.*, 2005): Pond 1 = 71·2 g; Pond 2 = 79·3 g; Pond 3 = 89·3 g.

Before introduction of *L. gibbosus*, eight 125 mL water samples were collected from each pond at eight equidistant locations around the pond's banks using a 183 cm sampling pole with a polypropylene sampling cup attached (Camlab Ltd, Cambridge, UK). The ponds were re-sampled in the same manner at 1, 3, 6, 12, 24 and 48 h intervals after introduction of *L. gibbosus* (these sampling intervals informed by the results of the previous tank trials). The sampling cup was moved in a standardised manner from the bank to the greatest extent reached by the pole, and vertically through the water in such a way as to obtain a sample representative of the entire water column. The water samples from a pond were combined in a sterilized 1 L plastic bottle. Between samples, the sampling pole and cup were disinfected using Microsol 3+ (Anachem Ltd, Luton, UK) and washed with de-ionised water. A plastic bottle of de-ionised water was taken into the field and processed identically to the pond samples, to act as a contamination control.

The water samples were immediately refrigerated at 4° C, and filtered as soon as practicably possible, within 72 h of collection. The pond water was pre-filtered through a 200 μm filter to remove coarse material, and then two sub-samples of 15 mL were filtered through polycarbonate filters of 0·4 μm pore size (Isopore, EMD Millipore, Darmstadt, Germany). This sample volume was determined by the amount of water which could practicably be filtered before clogging occurred. The two filters were placed in a 2mL tube and the DNA was extracted using the MO BIO PowerWater DNA Isolation Kit. PCR

amplification was as described previously, with the exception of the use of 6 μL of sample template.

FIELD SURVEY

For the detection of *P. parva* in natural conditions, water samples were collected from three ponds on a decommissioned ornamental fish farm, at the original introduction site of the species to the UK. (Gozlan *et al.*, 2002). Surveys using conventional sampling techniques (e.g. fish traps) had previously shown these ponds to contain *P. parva* populations at different densities (Pond 1, low density, 52 m × 7 m; Pond 2, intermediate density, 65 m × 15 m; Pond 3, high density, 52 m × 7 m). Fish community composition (non-target species) varied in these ponds, with no other species known from Pond 1; *T. tinca*, common carp *Cyprinus carpio* L. 1758, ide *Leuciscus idus* (L. 1758) and three-spined sticklebacks *Gasterosteus aculeatus* L. 1758 in Pond 2; and *G. aculeatus* in Pond 3.

From each pond, six 1-L water samples were collected from equidistant points around the pond's bank. Samples were transported to the laboratory on ice (in addition to a plastic bottle of de-ionised water handled identically to act as a control against contamination), refrigerated at 4° C and then filtered as soon as possible, within 24 h of their collection. Water was pre-filtered as described above, then an 80 mL sub-sample was filtered, the DNA extracted and PCR-amplified as described above.

RESULTS

The tests of primer sensitivity, on DNA extracted from fish tissue, revealed that the lower limit of detection varied between the four species. The lowest DNA quantity at which each species was reliably detected (i.e. producing a distinct gel electrophoresis band on all three PCR replicates), was: *L. gibbosus* = $4{\cdot}6 \times 10^{-4}$ ng; *L. delineatus* = $4{\cdot}6 \times 10^{-4}$ ng; *P. promelas* = 3×10^{-2} ng; *P. parva* = $1{\cdot}5 \times 10^{-2}$ ng.

In the aquarium trials, the eDNA of all four species, at all three levels of fish biomass, was detectable at the first sampling interval, i.e. 24 h following the addition of fish to the tanks (Table II), in all three PCR replicates. All control samples, collected before introduction of the fish, were negative for DNA of the target species, as were the de-ionised samples run through the equipment during filtering to test for contamination. The eDNA remained detectable for the remainder of the five-day experiment.

In the experimental pond trials, *L. gibbosus* eDNA was first detected in two of the three ponds 6 h after introduction of the fish. In the third pond, eDNA was first detected at 12 h following *L. gibbosus* introduction. In all three ponds, this result for time of first detection was confirmed by all three PCR replicates undertaken. *Lepomis gibbosus* eDNA was detected in all subsequent samples from all ponds for the remainder of the 48-h trial. All control samples collected before fish introduction proved negative for *L. gibbosus* DNA, as did the de-ionised water taken into the field as a contamination control.

In the field survey, *P. parva* eDNA was detected in all three of the decommissioned fish farm ponds where it was known to occur. However, the distribution of eDNA in all three ponds was spatially heterogeneous (Table III), with DNA detected at one location only in survey Ponds 1 and 2 (with low and intermediate *P. parva* densities, as indicated by previous trapping surveys) and at four locations in survey Pond 3 (high *P. parva* density detected in previous trapping surveys). No features of the pond were observed that were likely to account for the differences between sites of positive and negative detections. The de-ionised water taken into the field to act as a contamination control proved negative for *P. parva* DNA.

DISCUSSION

316 The present study demonstrates that conventional PCR methods can reliably detect the
317 presence of non-native fishes in water samples (Tables II-III), providing an effective means
318 with which environmental managers can map non-native species' distributions and thus
319 inform management decisions. This enables surveys to be undertaken in circumstances where
320 quantitative PCR (qPCR) facilities are not available or are financially prohibitive. Compared
321 to conventional PCR, use of qPCR methods increases the costs of materials required for
322 sample processing (× 1·5–2) and the cost of initial machine purchase (× 4).

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325 Whilst conventional PCR methods have been used successfully to survey aquatic
326 organisms (e.g. Deiner & Altermatt, 2014; Janosik & Johnston, 2015), many recent eDNA
327 trials (e.g. Barnes *et al.*, 2014; Pilliod *et al.*, 2014) and field surveys (e.g. Eichmiller *et al.*,
328 2014; Tréguier *et al.*, 2014; Gustavson *et al.*, 2015) use qPCR. One reason for considering
329 use of quantitative PCR would be the increased sensitivity reported in published studies,
330 compared to that achieved in the present study. Examples include detections of red swamp
331 crayfish *Procambarus clarkii* (Girard 1852) DNA at concentrations of 10^{-8} ng μL^{-1} (Tréguier
332 *et al.*, 2014), and newt DNA at quantities of 3×10^{-9} ng (Biggs *et al.*, 2015). The present
333 results demonstrated reliable detection at DNA quantities of 10^{-2}–10^{-4} ng (DNA extracted
334 from tissue). Importantly, detection at these DNA quantities proved sufficient to detect the
335 target species in the present study, at relatively low densities (in the *L. gibbosus* pond trials, a
336 mean biomass of 80 g in a pond of ≈ 25750 L). A study comparing conventional PCR and
337 qPCR found no difference in ability to detect DNA presence at low target organism densities
338 in mesocosm experiments (Nathan *et al.*, 2014), although further work would be needed to

determine how the detectability of target eDNA in field surveys in different environments relates to the sensitivity of the PCR protocol used.

These results emphasise the need to adapt sampling methodologies to the study site and the target species; this is to ensure sufficient spatial coverage of the water body to detect species of random or patchy distribution. Positive detections at only some of the sampling points in the field survey (Table III) suggested that the distribution of *P. parva* in these narrow ponds was relatively heterogeneous, as is often observed both temporally and spatially in field studies using fish microhabitat-orientated sampling approaches (Copp, 2010). Indeed, raw data collected for the purposes of another study revealed that in four size-classes of *P. parva* inhabiting the stream downstream of the disused fish farm (Beyer *et al.*, 2007), their dispersion index values were 0·004 to 0·021, indicating random distribution. In addition, experiments with *P. parva* in artificial streams have also indicated that native populations exhibit clumped/patchy distributions in response to available food and predator presence (Sunardi & Manatunge, 2005). Similar patterns of patchy eDNA distribution have been reported for lake-dwelling common carp *Cyprinus carpio* (L. 1758) (Eichmiller *et al.*, 2014). This patchiness in eDNA distribution emphasises the need for statistically-robust sampling protocols when attempting to determine presence/absence, taking into account both spatial heterogeneity due to habitat features (e.g. sediment, vegetation structure) and temporal aspects (e.g. seasonal movements of fish in relation to water temperature).

362 The quantity of water filtered in the current study, in both the pond trials (30 mL) and
363 field survey (80 mL), was lower than used in many eDNA studies (reviewed by Rees *et al.*,
364 2014b), and was selected as being the maximum water volume that could be filtered before
365 the membrane became clogged. Previous studies to determine the presence of non-native fish
366 in still water bodies have filtered 1–2 L (Takahara *et al.*, 2012, 2013; Moyer *et al.*, 2014), or
367 200 mL (Eichmiller *et al.*, 2014), whereas those studies that sampled quantities of water
368 similar to those in the present study (e.g. 15 mL) applied precipitation methods instead of
369 filtration (Ficetola *et al.*, 2008; Dejean *et al.*, 2012). These two methods (filtration and
370 precipitation) may yield different results in relation to detectability of eDNA, and indeed a
371 recent comparative study found a higher diversity of eukaryotic eDNA in 15 mL samples of
372 lake and river water using a filtration protocol than when using precipitation (Deiner *et al.*,
373 2015). The present results demonstrated that filtration of small sub-sampled volumes of water
374 can provide sufficient amounts of eDNA to detect the target fish species at the population
375 densities and waterbody sizes employed or encountered during the current study. Further
376 experimental studies would be needed to determine whether the variable rate of detection at
377 locations within ponds in the field survey would have been resolved (i.e. more positive
378 detections) with filtration of larger volumes.

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381 In field studies, the volume of water that can be filtered before clogging of the filter
382 occurs will depend on water character and filter pore size. The 0.4 μm pore size used in the
383 present study was similar to that used in many eDNA studies (Rees *et al.*, 2014b) but smaller
384 than that used in studies of non-native freshwater fishes in still waters, i.e. 3·0 μm (Takahara
385 *et al.*, 2012, 2013, 2015) and 1·5 μm (Eichmiller *et al.*, 2014). A recent study found that

386 common carp eDNA occurred in particles ranging from >180 μm to <0·2 μm, with eDNA
387 most abundant in particles of 1–10 μm, and recommended a filter of 0·2 μm pore size to
388 optimise collection of target DNA relative to total DNA (Turner *et al.*, 2014). In the present
389 study, use of 0·4 μm pore filters proved effective in capturing enough DNA for species
390 detection.

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393 Detection of fish in the present study was rapid, with successful detection within 6–12 h
394 after *L. gibbosus* were released into the experimental ponds, and in the initial samples (at 24 h
395 post release) in the tank experiments (Table II). Given the much smaller volumes of water in
396 the aquaria, it is likely that detection would have been considerably earlier if sampling had
397 been initiated prior to the 24 h interval. When a fish is introduced into a new environment
398 (tank or pond), it may be expected to release more DNA initially as a consequence of
399 elevated stress responses (Takahara *et al.*, 2012). Laboratory trials with Idaho giant
400 salamanders *Dicamptodon aterrimus* (Cope 1867) found an increased rate of eDNA
401 production in the first 2 h of aquarium occupancy, which the authors attributed to
402 physiological stress (Pilliod *et al.*, 2014). Environmental DNA released immediately upon
403 fish introduction in both tank and pond trials would have been expected to persist for the
404 length of the experiments, as previous studies have shown eDNA persistence (>5%
405 probability of detection) for 25 days in tanks and 17 days in ponds (Dejean *et al.*, 2011).

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In the tank experiments, positive detections were recorded at 24 h post release regardless of fish density (one, five or 10 fish), whereas the field survey yielded more detections in the pond containing the higher density of the target species. Most work to date has used environmental DNA surveys to determine the presence or absence of species, but management decisions could be better informed with information on the biomass of the target species. A series of trials in both laboratory tanks and experimental ponds with varying numbers of *C. carpio* did reveal a strong correlation between fish biomass and eDNA concentration (Takahara *et al.*, 2012), although some field surveys have found weak relationships between target species abundance (assessed using traditional survey methods) and eDNA concentration (Biggs *et al.*, 2015; Spear *et al.*, 2015), perhaps due to highly variable rates in the shedding of DNA within-species, and even by the same individual (Klymus *et al.*, 2015). Thus, further work is required before eDNA can be used reliably to determine the biomass of target species in field surveys.

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