

Article

A Brief Review of Non-Avian Reptile Environmental DNA (eDNA), with a Case Study of Painted Turtle (Chrysemys picta) eDNA Under Field Conditions

Clare I. M. Adams^{1,*}, Luke A. Hoekstra², Morgan R. Muell³ and Fredric J. Janzen²

- Department of Anatomy, University of Otago, Dunedin, Otago 9016, New Zealand
- 2 Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA 50010, USA; lhoek@iastate.edu (L.A.H.); fjanzen@iastate.edu (F.J.J.)
- 3 Department of Zoology, Southern Illinois University, Carbondale, IL 62901, USA; morgan.muell@siu.edu
- * Correspondence: clare.adams@postgrad.otago.ac.nz; Tel.: +64-022-012-9075

Received: 26 February 2019; Accepted: 26 March 2019; Published: 29 March 2019



Abstract: Environmental DNA (eDNA) is an increasingly used non-invasive molecular tool for detecting species presence and monitoring populations. In this article, we review the current state of non-avian reptile eDNA work in aquatic systems, and present a field experiment on detecting the presence of painted turtle (Chrysemys picta) eDNA. Thus far, turtle and snake eDNA studies have shown mixed results in detecting the presence of these animals under field conditions. However, some instances of low detection rates and non-detection occur for these non-avian reptiles, especially for squamates. We explored non-avian reptile eDNA quantification by sampling four lentic ponds with different densities (0 kg/ha, 6 kg/ha, 9 kg/ha, and 13 kg/ha) of painted turtles over three months to detect differences in eDNA using a qPCR assay amplifying the COI gene of the mtDNA genome. Only one sample of the highest-density pond amplified eDNA for a positive detection. Yet, estimates of eDNA concentration from pond eDNA were rank-order correlated with turtle density. We present the "shedding hypothesis"—the possibility that animals with hard, keratinized integument do not shed as much DNA as mucus-covered organisms—as a potential challenge for eDNA studies. Despite challenges with eDNA inhibition and availability in water samples, we remain hopeful that eDNA can be used to detect freshwater turtles in the field. We provide key recommendations for biologists wishing to use eDNA methods for detecting non-avian reptiles.

Keywords: turtle; environmental DNA; eDNA; non-avian reptile; review; eDNA guidelines; Chrysemys picata; painted turtle; shedding hypothesis

1. Introduction

Monitoring changes in a target species, such as presence/absence in a given locality and abundance, is necessary to model future population trends [1]. Indeed, changes in population density and abundance have downstream demographic effects on range, metapopulation structure, and niche availability [2,3]. Stochastic environmental factors, anthropogenic pressures, or biotic interactions (e.g., disease, intrinsic growth and age class, fecundity, or predation) can change population density [4–8]. Thus, changes in population density can inform researchers about fluctuations in environmental or biotic conditions. For example, novel habitat created by human activities could increase food resources, thereby expanding the area in which energy requirements can be met [9,10]. Increased resources then allow for an increase of population size [11]. Thus, monitoring current species presence and abundance may aid in predicting future densities if current population trajectories can be established.



1.1. Environmental DNA Uses and Limitations

Central to population monitoring is the need for a sensitive detection method. Recently, environmental DNA (eDNA) has received attention for being able to sensitively reveal the presence of target species, especially where traditional methods fall short [12,13]. We adopt Taberlet's (2018) definition of eDNA as DNA extracted from environmental samples such as soil, water, air, and feces [14]. Focusing on aquatic systems, single-species eDNA has been used in a few main ways for conservation: routine monitoring, detecting invasive species, and monitoring endangered species. Invasive amphibian species have also been targeted [15–17], as have crustaceans [18–22], reptiles [23,24], and molluscs [25–27]. Many endangered and secretive taxa have been detected using eDNA [28–35]. Recently, eDNA-obtained haplotypes have helped identify relatedness between endangered populations [36,37]. This non-invasive technique may even require fewer sampling permits compared to traditional methods, which can be difficult to obtain for protected species [38]. Environmental DNA has become an attractive method because of its sensitivity and efficiency [39–41], although this is not always the case [42]. A considerable time and money investment may be necessary

to establish an eDNA assay. However, this genetic method offers an advantage for identifying cryptic target species or species with small larval stages, which may be difficult even for expert taxonomists to identify [43–45]. These benefits of eDNA detection could provide managers with important information on population presence, thereby aiding initial monitoring and conservation efforts.

Although monitoring populations with eDNA methods has clear benefits, the utility of the information obtained from eDNA surveys beyond detecting species presence currently has limits. Many measures of diversity (e.g., most biodiversity indices) require abundance measurements, not simply presence [46]. Biomass can correlate with both sequence reads and eDNA copy number/concentration, but these relationships may be species- and ecosystem-specific and may not be clear [47–53]. Variable rates of eDNA shedding between individuals, diet, breeding season, and life stage may influence eDNA availability in the water column [52,54]. The environment also may play a role in eDNA degradation and capture [55–57]. In addition, false positives and false negatives must be carefully considered, requiring negative and positive controls [58–60]. Small sample size, insufficient replication, or lack of a sufficiently large sampling area can contribute to non-detection [13,59,61,62]. To mitigate this, species detection models [63] can be used to determine how many samples are necessary. Species occupancy models [64,65] can be used to determine the probability of presence. Both have been used for modeling eDNA detection.

1.2. Reptile eDNA

Despite breakthroughs in assessing density in fish and amphibian species, there remains a dearth of studies quantifying aquatic non-avian reptile populations with eDNA under field conditions [66]. This gap in the literature is notable because turtles are among the most at-risk vertebrates, with over 60% of modern species listed as threatened, endangered, or extinct [67,68]. To our knowledge, most eDNA studies on non-avian reptiles that heavily use aquatic habitats focus on detecting the presence of snakes (five studies) and turtles (nine studies) (Figure 1). An attempt was also made to find West African crocodile (*Crocodylus suchus*) and Nile monitor (*Varanus niloticus*) with eDNA metabarcoding methods, but presence has not yet been detected successfully [69]. For this study, 500 mL of water was filtered through a 0.45 µm nitrocellulose Whatman filter [69]. DNA was extracted with an ethanol precipitation and EZNA Tissue DNA kit and then amplified with the 12SV5.1 universal primer to target all vertebrates [69]. While human, dog, and teleost DNA were detected, reptile and amphibian eDNA were not [69].





Figure 1. A global map of non-avian reptile studies using eDNA and metabarcoding methods mentioned in this paper. Each color denotes a different study. Circles indicate snake studies and triangles indicate turtle studies. Note that one study by Lacoursière-Roussel et al., 2016 [70], found both snakes and turtles. Antarctica not pictured.

The first notable aquatic reptile eDNA study was on Burmese python (*Python bivittatus*) in South Florida [23]. These snakes inhabit semi-aquatic slough, coastal habitats, and marsh prairie [71]. This study extracted 15 mL water volumes from each site with a sodium acetate precipitation protocol and extracted eDNA with Qiagen's QIAamp DNA Micro Kit [23]. After successfully detecting python presence from aquatic eDNA using penned snakes, field sites with previously sighted pythons were tested [23]. Field sites yielded positive eDNA detection where *P. bivittatus* had been detected previously, and no eDNA was detected at one site where a python had not been detected previously [23]. Further research detected eDNA in terrestrial samples under field conditions in sites monitored via radio telemetry [66]. In this study, 950 mL of water was filtered using 0.45 µm cellulose nitrate filters near radio-tracked snakes [66]. This yielded a 58% detection rate using a quantitative PCR (qPCR) Taqman assay [66]. Another study confirmed these results by sampling sites with radio telemetry and burrow camera tracking [72]. However, unlike previous studies, soil samples were taken instead of water samples. Soil samples (~0.5 g) were extracted with a modified phenol-chloroform protocol and amplified with conventional PCR for *P. bivittatus* using a *CytB* primer [72]. Importantly, the study found eDNA degraded over time in soil, becoming undetectable four to seven days after snake presence [72]. This supports the use of soil eDNA tools for a temporally sensitive presence detection measure. Overall, eDNA can detect P. bivittatus in field settings, both for aquatic and terrestrial samples.

Additional aquatic snake studies have focused on the threatened eastern massasauga rattlesnake (*Sistrurus catenatus*) [73]. Fifty mL of water was taken from crayfish burrows, typical *S. catenatus* overwintering refugia, in occupied field sites [73]. Samples were centrifuged and filtered with a 1.4 μ M cellulose acetate filter, extracted with a Qiagen Qiashredder and Qiagen DNeasy Blood and Tissue kit, and run through the Zymo PCR Inhibitor Removal Columns [73]. A species-specific Taqman assay targeted the cytochrome oxidase I (*COI*) region of *S. catenatus* mitochondrial DNA (mtDNA) [73]. Despite known local abundance and collecting water within a meter of a snake, only two of 100 environmental samples amplified positively with eDNA, compared to 12 positive snake detections with traditional methods within a 2 m radius [73]. This assay was not effective in determining *S. catenatus* presence compared to visual confirmation.

Similarly, giant garter snake (*Thamnophis gigas*) eDNA assays were created for presence detection [74]. These snakes inhabit canals and marsh-like habitats [75]. In this study, laboratory

experiments detected *T. gigas* eDNA from tanks with snake skin and snake feces in water, but curiously eDNA was not detected in tanks with live snakes in the water [74]. This qPCR Taqman assay used 1 L water samples which were filtered with a 0.45 µm nitrocellulose filter, although precipitation with sodium acetate methods were also tested [74]. The Taqman assay amplified the *ND4* region of mitochondrial DNA (mtDNA), although cytochrome B (*CytB*) and NADH dehydrogenase 2 (*ND2*) regions were also considered [74]. *T. gigas* eDNA was not detected in water at field locations, despite capture with traps at the same sites [74]. Overall, this assay was not effective at detecting *T. gigas* eDNA in field settings compared to traditional methods.

With metabarcoding primers, redbelly snake (*Storeria occipitomaculata*), northern watersnake (*Nerodia sipedon*), and milksnake (*Lampropeltis triangulum*) eDNA presence was detected in Canadian lakes and rivers [70]. For this assay, 1 L water samples were filtered through 1.2 µm glass microfiber filters (Whatman GF/C), and DNA was extracted with the Qiagen QIAshredder and DNeasy Blood and Tissue kit [70]. Metabarcoding assays targeted the *CytB* and *COI* regions of mtDNA [70]. These results suggest that metabarcoding can effectively detect snake eDNA, although more studies are needed to confirm this finding. Overall, results have been mixed for detecting the presence of snakes with eDNA (Table 1) and, to our knowledge, no studies have yet attempted to quantify snake eDNA. It is possible that the more time snakes spend in water, the more likely aquatic eDNA will be able to detect snake presence; however, more research is needed to support this relationship.

| Study | Order | Species | Country | Laboratory | Field | Consistent Field — Detection? | Water Collection Details | Assay Type and Molecular Marker |
|-------------------------------|------------|---------------------------|---------|--|------------|--|---|--|
| | | | | Detection? | Detection? | | | |
| Baker et al., 2018 [73] | Squamata | Sistrurus catenatus | U.S.A. | - | Yes | 2/100 samples amplified with <i>S. catenatus</i> . | 50 mL of water filtered with a 1.4 μM cellulose acetate filter; DNA extracted with the Qiagen DNeasy Blood and Tissue kit | qPCR Taqman assay for COI |
| Cannon et al., 2016 [76] | Testudines | Terrapene carolina | U.S.A. | - | Yes | 2/91 samples amplified from universal amphibian primers. | 50 mL of water centrifuged; DNA extracted with the Qiagen DNeasy Blood and Tissue Kit | Metabarcoded with CytB, Illumina MiSeq 2 \times 250. |
| Davy et al., 2015 [24] | Testudines | Trachemys scripta | Canada | Yes | Yes | Yes, all PCR replicates of a field sample for <i>T. scripta</i> in a local pond. Other turtles not test for in a field setup. | 1 L of water filtered through 1.2 μm Whatman GF/C glass microfiber filters; DNA extracted with the Qiagen DNeasy Blood and Tissue Kit | qPCR SYBR (Intercalating dye) assay for COI |
| de Souza et al., 2016 [77] | Testudines | Sternotherus depressus | U.S.A. | Yes | Yes | Yes, four water samples required in the warm season and 14 water samples required in the cold season for a 95% detection probability. | 1 L of water filtered through a 0.80 μm cellulose nitrate filter; DNA extracted with the Qiagen DNeasy Blood and Tissue Kit. | qPCR EvaGreen (Intercalating dye) for 16S rDNA |
| Feist et al., 2018 [78] | Testudines | Macrochelys temminckii | U.S.A. | Yes | Yes | 2/3 to 1/6 replications amplified in the field when amplification occurred. | Between 850 and 1500 mL of water filtered through two 1.5 μm microfiber glass filters; DNA extracted with the DNeasy Blood and Tissue Kit | qPCR Taqman assay for CR of mtDNA |
| Halstead et al., 2017 [74] | Squamata | Thamnophis gigas | U.S.A. | Yes, limited. | No | No, no samples amplified. | 1 L of water filtered with a 0.45 μm nitrocellulose filter; DNA extracted with the MoBio PowerWater DNA isolation kit. | qPCR Taqman assay for ND4 |
| Hunter et al., 2015 [66] | Squamata | Python bivittatus | U.S.A. | Yes | Yes | Yes, 58% positive technical replicate detection near radio-tracked snakes | 950 mL of water filtered with a 0.45 μm cellulose nitrate filter and extracted with the PowerWater DNA Isolation Kit. | qPCR Taqman assay for ND4 |
| Kelly et al., 2014 [79] | Testudines | Chelonia mydas | U.S.A. | No—metabarcoding, yes—species-specific PCR | - | - | 1 L of water filtered with a 0.22 μm durapore membrane filter; DNA extracted with either the DNeasy Blood and Tissue kit or the PowerSoil DNA Isolation Kit | Metabarcoded with 12S rRNA gene or conventional PCR targeting CR of mtDNA |

Table 1. Studies that include research on snake or turtle environmental DNA in aquatic systems.

| Study | Order | Species | Country _ | Laboratory | Field | Consistent Field – Detection? | Water Collection Details | Assay Type and Molecular Marker |
|--|-------------------------|--|-------------|------------|------------|---|--|--|
| | | | | Detection? | Detection? | | | |
| Kucherenko et al., 2018 [72] | Squamata | Pantherophis guttatus, Python bivittatus | U.S.A. | Yes | Yes | 66.7% successful detection rate. | 0.5 g of soil extracted with a phenol-chloroform protocol | Conventional PCR with a <i>CytB</i> primer |
| Kundu et al., 2018 [80] | Testudines | Nilssonia nigricans, Nilssonia gangetica, Chitra indica | India | - | Yes | No information given on how many of the 10 replicates were successful. | 15–20 mL sodium acetate precipitation; DNA extracted with the QIAamp Tissue Extraction Kit | Conventional PCR with a COI primer |
| Lacoursiere-Roussel et al., 2016 [70] | Testudines, Squamata | Chelydra serpentina, Glyptemys insculpta, Nerodia sipedon, Lampropeltis triangulum, Storeria occipitomaculata | Canada | Yes | Yes | Yes, targeted qPCR detected wood turtle in 9/9 locations. eDNA metabarcoding detected two turtle species in 3/9 locations, but 4/9 metabarcoding locations did not detect wood turtle otherwise detected with qPCR methodology. Snake species were found in 3/9 locations. | 2 L of water filtered through a 1.2 μm glass microfiber filter (Whatman GF/C); DNA extracted with the QIAshredder and DNeasy Blood and Tissue Kit | qPCR Taqman Assay targeting the <i>COI</i> gene for the Wood Turtle; Metabarcoding using <i>CytB</i> and <i>COI</i> for non-turtle species. |
| Piaggio et al., 2014 [23] | Squamata | Python bivittatus | U.S.A. | Yes | Yes | Yes, 5/5 field sites with known presence amplified. | 15 mL sodium acetate precipitation; DNA extracted with the QIAamp DNA Micro Kit. | Conventional PCR for CytB |
| Raemy and Ursenbacher, 2018 [81] | Testudines | Emys orbicularis | Switzerland | Yes | Yes | 3/6 to 6/6 replications amplified in the field when amplification occurred. | 90 mL of water filtered through a 0.22 µm filter; DNA extracted with the QIAamp Tissue Extraction Kit | qPCR SYBR (Intercalating dye) assay for CytB |
| Wilson et al., 2018 [82] | Testudines | Batagur affinis | Malaysia | Yes | Yes | Yes, with live individuals within a 1 km vicinity of turtle presence. | 250 mL of water filtered through a 0.45 µm cellulose nitrate filter; DNA extracted with the NucleoSpin Tissue Kit | Conventional PCR with a <i>CytB</i> primer |

Table 1. Cont.

qPCR: quantitative PCR; COI: cytochrome oxidase I; CytB: cytochrome B; mtDNA: mitochondrial DNA.

Previous work has assessed the ability of eDNA to detect the presence of aquatic turtle species in a variety of habitats. In a marine aquarium, a green sea turtle (*Chelonia mydas*) was present but not detected when using eDNA metabarcoding methods [79]. Water samples were split into 1 L subsamples and filtered through 0.22 µm durapore membrane filters [79]. The filters were divided into groups, and DNA was extracted using either the DNeasy Blood and Tissue Kit or PowerSoil DNA Isolation Kit [79]. The vertebrate-specific *12S* rRNA gene was targeted for a metabarcoding assay, and the mtDNA *CR* of *C. mydas* was specifically targeted with conventional PCR [79]. While no turtle eDNA was detected with metabarcoding methods, the species-specific PCR assay detected the presence of *C. mydas*. This suggests species-specific assays may be more sensitive to detection of a particular species as compared to metabarcoding methods.

Similarly, eDNA assays were developed for multiple captive native Canadian turtles, and eDNA from red-eared slider turtle (*Trachemys scripta*) was successfully detected in a single artificial pond (Table 1) [24]. One-liter water samples were filtered with 1.2 µm Whatman GF/C glass microfiber filters and DNA was extracted out with the Qiagen DNeasy Blood and Tissue kit [24]. The *COI* region was targeted with a PowerSYBR qPCR assay [24]. All PCR replicates amplified, showing the ability to detect *T. scripta* presence in the field [24]. The increased testing of this assay in other bodies of water may help determine if this assay is more broadly applicable to other bodies of water. Importantly, this assay may help to detect where *T. scripta* has become an invasive species [83].

Additionally, an eDNA assay was developed to detect alligator snapping turtle (*Macrochelys temminckii*) presence in both lentic and lotic environments in the southeastern USA [78]. Filtered water volumes varied according to water sample clogging rates, so two 1.5 μ m microfiber glass filters were used to collect between 850 and 1500 mL of field water in total [78]. The Qiagen QIAshredder and DNeasy Blood and Tissue kits were used to extract out DNA which was amplified by a Taqman probe targeting the *CR* of mtDNA [78]. Technical replicates amplified 1/6 to 2/3 replicates, but technical replicates never amplified in all triplicate or sextuplicate reactions [78]. Furthermore, the replicates that did amplify had a high Cq value (>38), indicating a low eDNA concentration [78]. It is possible that turtle eDNA concentrations may approach the limit of detection in some cases.

In India, several imperiled turtle species (*Chitra indica, Nilssonia gangetica*, and *N. nigricans*) were detected in a temple pond using eDNA methodology [80]. Small water samples of 15–20 mL were collected and preserved with sodium acetate [80]. The DNA was extracted with the QIAamp Tissue Extraction Kit, and DNA was amplified with PCR targeting the *COI* region of turtle mtDNA (~650 bp), not specific to species [80]. Amplicons were sequenced to and confirmed to detect these three turtle species with BLAST [80]. This provides support that turtle species can be detected with eDNA, even with large amplicon sizes.

In Southeast Asia, the southern river terrapin (*Batagur affinis*) was detected in river samples in Malaysia [82]. Water samples (5 L) were collected close to a turtle nesting beach and 250 mL were filtered through a 0.45 µm cellulose nitrate filter [82]. The filters were kept in T1 lysis buffer and then extracted with the NucleoSpin Tissue Kit. Species-specific primers for *B. affinis* amplified the *CytB* region of the mtDNA genome with conventional PCR which yielded DNA [82]. This eDNA detection corresponded to the presence of at least one radio-tracked individual within 1 km (Table 1). This indicates that eDNA could be effective in detecting critically endangered turtles in their natural habitat. Potentially, this could aid in conservation efforts in Malaysia and other similar habitats.

Beyond presence detection, site-occupancy models in slow-flowing streams in the southeastern USA quantified the minimum number of eDNA samples needed to determine presence of the endangered flattened musk turtle (*Sternotherus depressus*) [77]. This study filtered 1 L of water through 0.80 µm cellulose nitrate filters [77]. Species-specific primers amplified part of the *16S* rDNA using an EvaGreen qPCR assay [77]. This study found the warm season (May–September) yielded higher eDNA detection rates for *S. depressus*, which likely corresponds to turtle activity [77]. Four replicate samples were needed in the warm season for a 95% detection probability versus 14 during the cool season. This supports the idea that sampling should be targeted to organism biology and active

periods. Additionally, it shows how many replicates may be needed to efficiently detect *S. depressus* under field conditions.

The density dependence of threatened European pond turtles (*Emys orbicularis*) in natural ponds was also investigated using eDNA in Switzerland [81]. To collect eDNA from ponds, 90 mL of water was filtered through a 0.22 µm filter, and DNA was extracted with the QIAamp Tissue Extraction Kit or sodium acetate precipitation methods [81]. A SYBR Green assay was used to amplify part of the *E. orbicularis CytB* gene. No correlation was found between turtle density, number, or biomass and eDNA abundance, although sites with vegetation and shallow waters yielded more turtle eDNA [81]. Detection probabilities ranged from 25–100%, and precipitation yielded increased detection (7 ponds versus 5 ponds) [81]. While unable to relate biomass to eDNA abundance, this study does support eDNA as a way to detect this threatened turtle under field conditions.

In Canadian riverine environments, the sensitivity of eDNA detection of at-risk wood turtles (*Glyptemys insculpta*) was tested [70]. As previously mentioned, 2 L water samples were filtered with 1.2 µm glass microfiber filters. However, qPCR Taqman assay targeted the *COI* region for *G. insculpta* abundance. The presence of *G. insculpta* was detected and correlated with turtle abundance from visual surveys. Furthermore, when using eDNA-metabarcoding methodology and universal primers, both *G. insculpta* and common snapping turtles (*Chelydra serpentina*) were detected. However, these metabarcoding methods did not detect *G. insculpta* eDNA in all rivers where qPCR eDNA methods detected this species [70]. This supports a trade-off between single-species and metabarcoding methods; Taqman qPCR assays may be specific and sensitive but limited to one species, while metabarcoding is less specific but can detect multiple species within a sample. Indeed, this is also seen in the aforementioned aquarium mesocosm study [79].

Finally, the eastern box turtle (*Terrapene carolina*) presence was detected using metabarcoding methods on an Illinois river, though turtle presence was not confirmed with an actual specimen [76]. Surface water (~50 mL) was sampled, centrifuged, and then extracted with the Qiagen DNeasy kit [76]. Twelve primers were used to amplify eDNA, of which the amphibian primer amplified a *CytB* region of the mtDNA genome, detecting *T. carolina* when analyzed after Illumina sequencing [76]. This study adds support that turtles can be detected with metabarcoding, despite previous attempts which have been less successful. However, metabarcoding likely can only detect presence [84]. Taken together, the total of these studies illustrates success in detecting turtle eDNA in aquatic systems, indicating promise for using this population monitoring technique in this increasingly imperiled group. However, work remains to be done in achieving consistent and sensitive detection, and obtaining eDNA quantities that reflect abundance.

1.3. Painted Turtle eDNA Case Study

At the conception of this experiment in 2015, essentially no turtle eDNA studies had been published (Table 1). Thus, we conducted a field experiment to quantify relationships between turtle density and turtle eDNA over time in a lentic pond system. We used painted turtles (*Chrysemys picta*) as a model because they exist in the same aquatic habitats as multiple endangered turtle species, such as the yellow mud turtle (*Kinosternon flavescens*) and Blanding's turtle (*Emydoidea blandingii*) [85]; insights into detecting painted turtle with eDNA could potentially provide instrumental knowledge for developing eDNA conservation tools for these sympatric endangered species. We populated semi-natural ponds with varying numbers of adult turtles and correlated painted turtle eDNA in water samples with painted turtle biomass in this enclosed system over a three-month period. We hypothesized the amount of total eDNA and turtle eDNA would linearly increase with time and turtle density. Establishing a relationship between eDNA concentration and turtle density between ponds and throughout time could deliver an eDNA-based monitoring tool for the painted turtle and other imperiled freshwater turtles.

2. Materials and Methods

2.1. Experimental Setup and eDNA Collection

We seeded four closed-system outdoor ponds with painted turtles at the Iowa State University Horticulture Farm in 2016. These outside, uncovered ponds were natural with respect to abiotic variables, and water was not treated in any way, so rainfall and evaporation were not controlled. It is possible the source water supply had turtle eDNA. We lined the ponds with black polyethylene laminated tarp and added three white water lily plants (*Nymphaea* sp.) to each pond. Ponds were surrounded by an electric fence, preventing foreign turtles from entering. Although these ponds were the same dimensions (19 m L × 15 m W × 1.5 m D each), they varied in number of adult turtles (0, 11, 23, 38) and initial biomass (0 g, 6088 g, 9198 g, and 12990 g, respectively). We labeled these ponds as zero (0 turtles at a density of 0 kg/ha), low (11 turtles and a density of 6 kg/ha), medium (23 turtles at a density of 9 kg/ha), and high (38 turtles at a density of 13 kg/ha) density. In North American aquatic systems, painted turtle densities can range between 7.2 and 106 kg/ha [86,87]. Our pond densities most mimic low-density painted turtle populations, as these would most likely be relevant to co-occurring imperiled species. We placed turtles in the ponds on 1 April 2016, which coincides with extensive painted turtle post-hibernation activity [88].

We sampled 250 mL of water at randomized locations around the perimeter of each pond approximately 0.75 m from the edge once every three days starting 1 April through 30 June 2016. A total of 120 pond samples were taken, one sample from each of the four ponds once every three days for three months. One sample was taken from every pond on each sampling day to ensure all samples could be processed in a timely manner. To process samples within 48 h, we chose small water sample volumes due to frequent filter clogging and high turbidity. We took samples in 10% bleach sterilized, autoclaved glass Nalgene jars. When sampling, we used sterile gloves and changed them between ponds. We did not touch the water's edge with our feet to prevent pond-to-pond contamination. No PCR products (amplicons) existed in the field, and samples were taken in the morning before any laboratory work. We immediately transported samples to Iowa State University, a 15 min drive without ice, stored them in a 4 °C refrigerator, filtered them, and extracted DNA within 48 h. Samples were filtered with 0.45 µm cellulose nitrate filters in a room never used for amplifying turtle DNA, inside a laminar flow hood (however, they were carried to a room with PCR products from past testudine and squamate experiments for extraction and amplification).

Painted turtle eDNA was also extracted from laboratory water containing captive turtles for use as an eDNA positive control ("turtle lab water"). Four adult turtles were placed in a bin (0.59 m \times 0.42 m \times 0.27 m; 47 L volume) about one-third full of water for two weeks during their hibernation period. Water was sampled as above on 15 January 2016 and immediately filtered using 0.45 μ m cellulose nitrate filters in a room never used for amplifying turtle DNA. Turtle lab water was never handled in the same week as pond eDNA water.

2.2. Extraction

We optimized our eDNA protocol by testing multiple published eDNA methods and commercially available extraction kits before settling on the following methods. We processed all samples under a UV-sterilized hood to ensure sterility. Between all samples, extraction equipment (a Buchner funnel, tweezers, glass Nalgene jars, and a large Erlenmeyer flask) were rinsed with 10% bleach and rinsed three times with deionized water. Gloves were changed between samples. Negative controls (250 mL of diH₂O) were taken haphazardly throughout the experiment. We vacuum-filtered water samples through a 0.45 μ m-pore cellulose nitrate filter. Once filtration was finished, we immediately folded the filter inward and put it into a QIAshredder with 350 μ L of buffer ATL and 25 μ L of proteinase K [17,26,89–91]. We then incubated the sample overnight at 65 °C [92,93]. After the overnight incubation, we spun down the QIAshredder column for 2 min at 14,000 rpm and added 200 μ L of buffer AL and 200 μ L of 95% ethanol to the elute. After vortexing, we put the solution into a DNeasy

Blood and Tissue Kit spin column and spun the sample in a microcentrifuge for 2 min at 14,000 rpm [26]. We followed Qiagen's Manufacturer's instructions starting with the addition of 500 μ L of Buffer AW1 (Step 5) until elution (Step 7). We eluted the samples with 200 μ L of ethylenediaminetetraacetic acid (EDTA) (low tris EDTA (TE)) buffer heated to 65 °C [24]. We also filtered and extracted three negative laboratory control samples using Culligan Nanopure water in this same way.

2.3. Amplification and Quantification

No species-specific qPCR protocol existed at the time of sampling for the painted turtle; therefore, we developed our own. Thermo Fisher Scientific (Waltham, MA, USA) designed a primer-probe combination from painted turtle mtDNA, COI, using GenBank Accession numbers KF874616.1, NC_023890.1, NC_002073.3, and AF069423.1. Primer and probe sequence can be ordered using Taqman Assay APMFWY7_C_PICTA_V2 from Thermo Fisher Scientific. These were custom designed to have at least six mismatches over both primers and probe from five other sympatric turtle species (Chelydra serpentina (GenBank Accession Numbers EF122793.1, NC_011198), Trachemys scripta (GenBank Accession Numbers NC_011573.1, FJ392294.1), Apalone spinifera (GenBank Accession Numbers NC_021371.1, JF966197.1), Graptemys ouachitensis (GenBank Accession Number JN993985.1 (incomplete mtDNA genome), and Graptemys geographica (GenBank Accession Number JN993982.1 (incomplete mtDNA genome)). We tested species-specificity of the primer/probe set by amplifying DNA from blood samples from these five sympatric turtle species. These turtle species and negative controls all yielded quantification cycle (Cq) values ≥ 5 higher than painted turtle amplification, denoting species specificity [94,95]. Due to cost and time constraints, we ran a subset of our field samples, using samples from all ponds from dates spaced at roughly two-week intervals: 31 March, 16 April, 1 May, 16 May, 31 May, 15 June, and 30 June.

We performed a qPCR assay composed of 20 μ L of PerfeCTa qPCR ToughMix (Quanta Biosciences, Beverly, MD, USA), 10 μ L of nanopure water, 2 μ L of the Taqman primer/probe reaction mix, and 8 μ L of 1:4 diluted template for a final reaction volume of 40 μ L. Reaction conditions were as follows: 10 min initial denaturation at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. We ran qPCR reactions in triplicate and averaged the Cq values for each sample. We ran a dilution series using DNA extracted from painted turtle blood and painted turtle eDNA from laboratory water in a 1:2 dilution series. We ran one sample (31 May, high-density pond) alongside the same dilutions. Due to non-linear eDNA amplification likely from an inhibitor presence, we chose a 1:4 dilution for all samples [96]. Using more concentrated eDNA consistently failed to improve eDNA amplification, indicating the presence of inhibitors [96].

We assumed that replicates that did not return a Cq value were below detection limit and excluded them from Cq averages, standard deviation (SD), and standard error of the mean (SEM) for the sample. Samples without Cq values also were excluded from future analysis. All qPCR runs contained no template controls in triplicate, and all were prepped in a UV-sterilized hood treated with 10% bleach. We only considered values <33 Cq to ensure our samples were distinct from background amplification [94,95]. Assuming exponential amplification, less than 1% (0.95%) of signal contribution would be non-target DNA contribution when efficiency is 100% ($10^{-1/m}$, m = -3.497 = slope of eDNA lab water standard curve, E_{AMP} = 1.932, intercept = 25.888) [94].

In addition to assessing absolute Cq values, we examined the ordered trend of the lowest Cq value to the highest Cq value among ponds and controls, with abundance corresponding to 1/Cq. Thus, we expected the pond with the highest turtle density to have the lowest Cq value followed by ponds with medium, low, and zero densities of turtles. We also included positive controls (DNA extracted from blood and turtle laboratory water) and negative controls, expecting extracts from blood to have the highest concentration of turtle DNA, followed by turtle lab water and the negative controls. We evaluated the statistical significance of this ordering with Jonckheere's trend test. This test is similar to the Kruskal–Wallis test, but is used specifically to assess a priori ordering hypotheses [97]. Our null hypothesis was that there was no trend order, whereas our alternative hypothesis dictated the following

strict trend: turtle blood, turtle laboratory water, high-turtle-density pond, medium-turtle-density pond, low-turtle-density pond, zero-turtle-density pond, and then the negative controls. To perform these tests, we used the packages *clinfun* and base R statistical software and plotted the data with *ggplot2* (version 3.2.3) [98].

This study was performed in accordance with Iowa State University Care and Use of Animals Protocol #12-03-5570-J.

3. Results

Our dataset was comprised of seven sampling days; triplicate qPCR values were averaged to yield 27 Cq values. One sample—from the zero-turtle-density pond on 15 June—was below our detection limit and did not yield a Cq value. As this pond had no turtles, we expected this pond to not yield turtle eDNA. Our negative controls amplified at an average Cq of 40.07 (SD = 0.39, SE = 0.11) and our blood positive control Cq was 21.43 (SD = 0.39; SE = 0.11). Background signal in the negative controls was always detected. The lowest mean value (i.e., highest eDNA abundance) for any sample was the high-turtle-density pond on 1 May, with 31.06 Cq (SD = 0.39; SE = 0.11). This reading is more than 7 Cq values away from the mean of our negative controls, rendering it able to be considered for analysis [94]. The next highest eDNA abundance was for the medium-turtle-density pond on 15 June, with 33.92 Cq (SD = 0.08; SE = 0.04), which is not more than 7 Cq values away from the negative control and therefore not sufficiently distinguishable from background amplification. Thus, with only one sample meeting detection criteria, we could not statistically analyze individual Cq values (Figure 2). That we detected background signal, however, indicates that our amplification assay was sensitive and that potential turtle-specific eDNA signal, if present, would be lower than background signal.



Figure 2. Amplification (quantification cycle = Cq) of adult painted turtle eDNA as a function of sample source and date. Higher Cq values indicate less eDNA. Varying colors and symbols represent pond treatments: the zero-density pond had 0 turtles (orange squares), the low-density pond had 11 turtles (red circles), the medium-density pond had 23 turtles (purple triangles), and the high-density pond had 38 turtles (blue diamonds). Points indicate the average triplicate value of each sample and points are jittered for readability. The positive controls from extracted painted turtle blood and the negative controls were plotted at the left column to facilitate comparisons. The zero-density pond on 15 June failed to amplify, and only one replicate of the low-density pond on 31 March amplified.

Regardless of sample Cq values relative to background amplification, we assessed whether sampled Cq values followed an expected trend of turtle-specific eDNA concentrations. The rank-order obtained for highest to lowest amplification of turtle-specific eDNA was: turtle blood, turtle lab water, high-turtle-density pond, medium-turtle-density pond, low-turtle-density pond, zero-turtle-density pond, and our negative control (Figure 3). This ranking of turtle-specific eDNA concentrations exactly matched our alternative hypothesis, and Jonckheere's test suggested a meaningful order to these samples (p < 0.001).



Figure 3. Plot of Cq mean per sample source with the standard error of the mean (SE) for painted turtle eDNA from water samples obtained from experimental ponds during the 2016 field season. Higher Cq values indicate lower eDNA. Varying colors and symbols represent pond treatments: the zero-density pond had 0 turtles (orange squares), the low-density pond had 11 turtles (red circles), the medium-density pond had 23 turtles (purple triangles), and the high-density pond had 38 turtles (blue diamonds). See Figure 2 for more information.

4. Discussion

Overall, we could not discern quantitative patterns of painted turtle-specific eDNA in individual samples from semi-natural ponds, indicating potential detection limitations. This result occurred despite known abundances of turtles in the water we sampled and a sensitive qPCR assay. While our results are clearly false negatives (Type II error), they also may demonstrate the technological limits of quantifying abundance of freshwater turtles in the wild using similar eDNA techniques, even given considerable effort to troubleshoot and optimize system-specific protocols. We conclude that our qPCR protocol for painted turtle-specific eDNA did not effectively quantify turtle density, because only 1 of 27 field samples amplified substantial turtle-specific eDNA (the high-density pond on 1 May). Cq values generally followed the rank-order predictions based on abundance, but lack of sufficient separation from background signal prevents further interpretation of these differences.

We developed an eDNA amplification assay for detecting and quantifying turtle eDNA. We detected background painted turtle signal despite thorough use of UV-sterilizing equipment before qPCR amplification, isolation of qPCR preparation from DNA extraction, and much care to prevent contamination. Although the majority of our turtle-specific eDNA samples did not differ enough from the persistent background noise to allow quantitative analysis, the raw abundances do qualitatively follow the expected rank-order pattern from highest-turtle-density pond to lowest-turtle-density pond. We emphasize the need for PCR-free environments when filtering, extracting, and preparing PCRs when working with eDNA samples. By ensuring these working environments, negative controls have less of a chance of being contaminated. If we had detected a higher concentration of painted turtle eDNA in our samples or no contamination in our negative results, we would have expected to have obtained enough copies of eDNA for quantitative analysis. If we had found differential amplification from each of the seeded ponds, then turtle densities as well as turtle number and biomass would need to be considered as potential explanations of varying eDNA.

Turtle eDNA possibly has a stochastic nature at low concentrations, exemplified by one clear amplification and several others that fall short of the cutoff (Figure 2). Other turtle studies have found inconsistent amplification as well [78,81]. Inconsistent amplification occurs with other low-density species [16,21]. Larger water samples passing through multiple filters may have mitigated this issue by increasing the chance of turtle eDNA capture [99]. However, while larger water samples may increase target species eDNA abundance, inhibitor abundance may also increase. To guard against the stochastic nature of low-concentration eDNA, appropriate replicates should be taken [100]. We have taken replicates over time, but the study may have benefitted from multiple samples from each pond on the same day as well as over time. This may have increased our chances at detecting turtle eDNA distinct from background noise. Furthermore, if time and finances allowed, we would have increased qPCR replicates and recommend more replicates for future studies. However, if eDNA is to be widely used for routine monitoring, inclusive of institutions which do not receive much funding or may be resource limited, a robust assay which uses less than the desired number of replicates may be the only option. Despite an abundance of turtles in the sample water, we were unable to collect and extract enough turtle eDNA to reliably exceed the detection limit of qRT-PCR.

Currently, we cannot recommend our particular eDNA quantification assay for monitoring aquatic turtle density under field conditions. We obtained just one substantially amplifiable sample of turtle eDNA from pond water despite successfully amplifying turtle-specific eDNA from lab water and developing a sensitive qPCR amplification assay. On the other hand, we did observe the expected positive relationship between turtle density and turtle-specific eDNA, hinting at a possible correlation between turtle density and eDNA extracted. Still, this study highlights some limitations of detecting aquatic reptile eDNA density under field conditions. Other studies have also reported similar difficulties of not being able to relate known turtle density to eDNA under field conditions [81]. Still, advances in technology may soon realize the full potential of eDNA for monitoring the density of turtle populations. One avenue is ddPCR, a sensitive PCR tool that absolutely quantifies template copy number [48,49,101]. This technology has already shown a correlation between density and eDNA copy number in a variety of environments and could be used to aid in quantifying reptile eDNA [49]. As ddPCR technology becomes more widely available and decreases in cost, it may be an attractive alternative to current qPCR methods, especially as it can be more robust to inhibition than qPCR [102,103]. That fish and amphibians have well developed eDNA techniques lends optimism to the view that eDNA eventually can be used to monitor populations of aquatic turtles.

4.1. Inhibition

As with other eDNA studies, our experiment likely suffers from DNA inhibition in the environmental samples. When dilutions were run, 1:4 and 1:8 sample dilutions had a lower Cq value than the full sample itself, signaling the presence of inhibitors [96]. With non-inhibited DNA extracted from painted turtle blood and painted turtle laboratory water, this was not the case. Despite the troubleshooting with Environmental Master Mix 2.0 and the use of ToughMix (Quanta Biosciences), specifically designed to reduce the effects of PCR inhibition, we were unable to amplify enough turtle eDNA to quantitatively relate to turtle density. Inhibition is common in eDNA field studies and is addressed through various protocols. Employing special buffers during extraction

(e.g., cetyltrimethylammonium-bromide CTAB), applying clean-up kits (e.g., Zymo One Step), using BSA in PCR reactions, and diluting template for PCR reactions are common ways of minimizing the effect of inhibitory compounds [24,104–107]. Common environmental inhibitors include plant secondary compounds such as polysaccharides, pectin, xylan, phenols, and tannins [108,109]. Soil also contains known PCR inhibitors including humic acids, minerals such as calcium, and inorganic compounds [108,109]. Proteases, urea, and competing DNA may additionally inhibit reactions or decrease reaction efficiency [109]. Furthermore, the addition of a pre-filtration step may aid in capturing non-target particles for an easier second filtration step [110]. While inhibitors are well documented in the literature, it may be difficult to ascertain exactly what mixture of inhibitors are responsible for decreased PCR yield. Therefore, general methods such as clean-up kits and dilution are commonly used for eDNA samples.

4.2. The Shedding Hypothesis

Biologically, non-avian reptiles may not shed eDNA into the environment at the same rates as other organisms. This we dub the "shedding hypothesis," or the hypothesis that organisms with a keratinized exterior integument may shed eDNA at lower rates compared to those with a mucus integument, such as teleost fish and amphibians. For example, turtles lack gills, and most integument is keratinized, so they may not shed eDNA as readily as organisms with a mucus layer [85,111]. Indeed, one study noted that, when eDNA metabarcoding is used for non-avian reptiles and amphibians, >95% of read abundance was comprised of amphibian DNA for that specific primer set [70]. Potentially, amphibian DNA is more abundant in environmental samples than non-avian reptile eDNA and thus contributes to a larger percentage of read abundance. Furthermore, turtles commonly shed scutes and skin in pieces (rather than as rafts of cells), which, due to their mass, may sink into substrate and be unlikely to be detected in the water column as readily by our eDNA methodology [112]. Thus, only excrement, tears, and saliva may be primary shedding mechanisms for detecting turtle eDNA [113,114]. As a result, turtle eDNA may not be overly abundant in the water column. For example, when detecting alligator snapping turtle (*M. temminckii*) presence, Cq values were larger than the usual <35 Cq, ranging from 39.06 to 44.89 Cq, indicating low quantities of eDNA [78]. Additionally, despite detection, that study had a low rate of replicates amplifying in a field setting, with most amplifications occurring at a 16–33% rate with no 100% replication rates [78]. In studies of European pond turtles, some ponds with known turtle presence did not yield eDNA, resulting in false negatives [81]. Further evidence comes from a previous mesocosm study, specifically targeting marine vertebrates in a semi-controlled environment, where no turtle eDNA was found with vertebrate metabarcoding primers, although a sea turtle was present [79].

Along with turtles, other animals with hard exteriors may have reduced shedding of eDNA. For example, European green crab eDNA (*Carcinus maenas*) was about an order of magnitude lower than that of shanny fish eDNA (*Lipophrys pholis*) in a laboratory marine setup, despite comparable biomass added to tanks [57]. Shedding of large skin fragments, rather than numerous small bits of tissue containing DNA, also may have contributed to non-detection in previous studies of non-chelonian reptiles in aquatic systems. Despite the aquatic nature of West African crocodiles (*C. suchus*), the species was not detected in a metabarcoding study [69]. Additionally, giant garter snake (*T. gigas*) individuals placed in water were not detected with eDNA in a laboratory setting, suggesting that live snake presence may not be enough to shed sufficient eDNA [74]. However, given that substances such as fecal matter can yield DNA [74] (and some successful snake detections have occurred using eDNA in the wild [23,70]), animals with non-mucus integument ultimately may be detectable via eDNA sources other than skin. The shedding hypothesis presented here may be applicable beyond turtles and other vertebrates with keratinized skin, but likely only reduces environmentally available DNA rather than prevents eDNA shedding altogether. Thus, shedding rates may need to be analyzed for this group of animals, as compared to that of fish or molluscs [50,115]. We

present the shedding hypothesis as just one potential explanation for why eDNA may be less available in the water column for organisms with relatively rigid exteriors.

4.3. Best Practices

Both the system and the particular target should be considered when sampling. Different targets require different considerations. It is usually best to carry out a small-scale proof-of-concept experiment in conjunction with traditional methods for comparison before widely applying eDNA methods for monitoring. Here, we outline a few considerations when designing a species-specific eDNA study and recommend additional reviews of eDNA study design [116,117].

Before obtaining samples, planning a robust experimental design as well as having a clean, DNA-free space where experiments will be carried out is important [116,118]. Target species' biology can be used to optimize sample timing. Periods of increased activity, such as breeding seasons, can elevate eDNA availability in the water [119,120]. For example, painted turtle eDNA may be taken while animals are not hibernating and during times of day when they are most active and not basking. Samples should be taken with an appropriate number of replicates [77], which may vary depending on season and target biology. Regardless, replicates may increase the chance of detection and confirm positive detection beyond stochasticity [77,100]. Field site(s) should also be considered, as eDNA travels downstream in lotic systems or can have different spatial distribution in lentic systems [47,121–124]. Water samples need to be filtered, be extracted, and have PCRs set up in a PCR-free room, preferably in another building, floor, or lab. These practices will prevent contamination, especially if the target species DNA has been amplified before in the same lab.

Beyond planning, sampling and laboratory workflows should be considered. Multiple negative controls (e.g., field, extraction, amplification, and sequencing) are needed to determine at what step contamination is introduced, if at all [60]. During amplification and sequencing, positive controls should be used for comparison, such as laboratory eDNA or DNA tissue extract from the target species [116,125]. At times, synthetic positive controls have been used to distinguish positive controls from potential contamination [33,125]. Furthermore, primers should be tested with closely related, sympatric species to ensure species specificity. Probe-based qPCR for closely-related taxa can increase amplicon specificity [126] to discern single base pair mismatches.

To obtain eDNA, many filtration, extraction, and amplification methods have been used. It may be best to test various filter types systematically, but protocols often use cellulose nitrate filters with 0.45 μM pores to capture eDNA [105,110]. A larger pore size may be needed if clogging occurs, especially with water containing high concentrations of algae or sediments [127]. Generally, larger volumes (>1 L) of water increase the chance of detecting organisms, though increasing replicates can allow for smaller volumes to be used [77,99,128]. Once filtered, samples are extracted, such as with Qiagen's Blood and Tissue Kit or via a phenol-chloroform isoamyl solution [105]. To decrease sample inhibition, the Zymo's One-Step PCR Inhibitor Removal Kit can be helpful, although dilution can work as well [96,129]. Both methods may decrease inhibition, but also potentially risk decreasing extracted DNA concentration or yield [129]. Turbid aquatic environments can be more prone to inhibition, yet it may still be possible to obtain eDNA from them [110,129]. To increase PCR reaction efficiency, bovine serum albumin (BSA) may also be added to PCR reactions [24]. Once successful, Sanger sequencing of amplicons can be used to confirm target species DNA. A number of positive identifications across replicates may be needed to support the presence of a target organism, depending on the habitats sampled (e.g., lentic, lotic, or marine) and how dilute the eDNA is expected to be [78,116].

5. Conclusions

Beyond solving eDNA technical difficulties, there is no stand-in for knowing the biology of the target organism. To maximize the probability of success of using eDNA, sampling should be targeted to the life history and ecology of the particular species. Without this basic research, genetic knowledge, and rigorous testing of methodology, eDNA monitoring may not easily yield useful results. As in

our case study, painted turtle eDNA may be difficult to obtain in the field. Even so, eDNA could be a powerful tool for detecting presence of non-avian reptiles in lentic habitats [130], as it is already being used successfully for fish and amphibians [131,132]. Although employing eDNA for studying reptiles in aquatic systems presents challenges, such as decreased eDNA shedding, we remain hopeful that more sensitive technological advancements and robust study design will mitigate these issues.

Author Contributions: Conceptualization: C.I.M.A. and F.J.J. Data curation: C.I.M.A. and M.R.M. Formal analysis: C.I.M.A. and L.A.H. Funding acquisition: C.I.M.A. and F.J.J. Methodology: C.I.M.A., M.R.M., and L.A.H. Project administration: L.A.H. and F.J.J. Visualization: C.I.M.A. and L.A.H. Writing—original draft preparation: C.I.M.A. Writing—review and editing: L.A.H., M.R.M., and F.J.J. Supervision: F.J.J.

Funding: This research was funded by grants from the National Science Foundation (DEB-1242510 and IOS-1257857) and the Wildlife Diversity Program of the Iowa Department of Natural Resources (15CRDWBKKINK-0075), a Gaige Award from the American Society of Ichthyologists and Herpetologists, and a Sigma Xi Grant-in-Aid of Research.

Acknowledgments: We are most grateful to Jack M. Gallup for help with qPCR troubleshooting. We are also grateful to Rachel Weber and Paige Koerperich for laboratory assistance, to Nick Howell et al. for permission and assistance with the ISU Horticulture Research Station, and to the Illinois DNR, the US FWS, and the ISU IACUC for permits. We thank the members of the Janzen lab for constructive criticism.

Conflicts of Interest: The authors declare no conflict of interest.

References

- 1. Caswell, H. *Matrix Population Models: Construction, Analysis, and Interpretation;* Sinauer Associates: Sunderland, MA, USA, 1989; ISBN 0878930930.
- 2. Wells, J.; Richmond, M. Populations, metapopulations and species populations—What are they and who should care? *Wildl. Soc. Bull.* **1995**, *23*, 458–462.
- 3. Holt, R.D. Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary perspectives. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19659–19665. [CrossRef]
- 4. Kendall, D.G. Stochastic processes and population growth. J. R. Stat. Soc. Ser. B 2018, 11, 230–264. [CrossRef]
- 5. Sutcliffe, O.L.; Thomas, C.D.; Moss, D. Spatial synchrony and asynchrony in butterfly population dynamics. *J. Anim. Ecol.* **2006**, *65*, 85. [CrossRef]
- 6. Saether, B.-E.; Bakke, O. Avian life history variation and contribution of demographic traits to the population growth rate. *Ecology* **2000**, *81*, 642. [CrossRef]
- 7. Frankham, R. Genetic adaptation to captivity in species conservation programs. *Mol. Ecol.* **2008**, 17, 325–333. [CrossRef] [PubMed]
- 8. Caswell, H. Matrix models and sensitivity analysis of populations classified by age and stage: A vec-permutation matrix approach. *Theor. Ecol.* **2012**, *5*, 403–417. [CrossRef]
- 9. Marzluff, J.M.; McGowan, K.J.; Donnelly, R.; Knight, R.L. *Causes and Consequences of Expanding American Crow Populations*; Marzluff, J.M., Bowman, R., Donnelly, R., Eds.; Springer: Boston, MA, USA, 2001; ISBN 9978-1-4613-5600-4, 978-1-4615-1531-9.
- 10. Mills, C.E. Jellyfish blooms: Are populations increasing globally in response to changing ocean conditions? *Hydrobiologia* **2001**, *451*, 55–68. [CrossRef]
- 11. Dobson, F.S.; Kjelgaard, J.D. The influence of food resources on population dynamics in Columbian ground squirrels. *Can. J. Zool.* **1985**, *63*, 2095–2104. [CrossRef]
- 12. Gargan, L.M.; Morato, T.; Pham, C.K.; Finarelli, J.A.; Carlsson, J.E.L.; Carlsson, J. Development of a sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: A case study of devil ray at seamounts. *Mar. Biol.* **2017**, *164*, 112. [CrossRef]
- 13. Tucker, A.J.; Chadderton, W.L.; Jerde, C.L.; Renshaw, M.A.; Uy, K.; Gantz, C.; Mahon, A.R.; Bowen, A.; Strakosh, T.; Bossenbroek, J.M.; et al. A sensitive environmental DNA (eDNA) assay leads to new insights on Ruffe (*Gymnocephalus cernua*) spread in North America. *Biol. Invasions* **2016**, *18*, 3205–3222. [CrossRef]
- Cilleros, K.; Valentini, A.; Allard, L.; Dejean, T.; Etienne, R.; Grenouillet, G.; Iribar, A.; Taberlet, P.; Vigouroux, R.; Brosse, S. Unlocking biodiversity and conservation studies in high-diversity environments using environmental DNA (eDNA): A test with Guianese freshwater fishes. *Mol. Ecol. Resour.* 2019, *19*, 27–46. [CrossRef] [PubMed]

- 15. Ficetola, G.F.; Miaud, C.; Pompanon, F.O.; Taberlet, P. Species detection using environmental DNA from water samples. *Biol. Lett* **2008**, *4*, 423–425. [CrossRef] [PubMed]
- 16. Dejean, T.; Valentini, A.; Miquel, C.; Taberlet, P.; Bellemain, E.; Miaud, C. Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog *Lithobates catesbeianus*. *J. Appl. Ecol.* **2012**, *49*, 953–959. [CrossRef]
- 17. Secondi, J.; Dejean, T.; Valentini, A.; Audebaud, B.; Miaud, C. Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA. *Amphib. Reptil.* **2016**, *37*, 131–136. [CrossRef]
- Ardura, A.; Zaiko, A.; Martinez, J.L.; Samulioviene, A.; Semenova, A.; Garcia-Vazquez, E. eDNA and specific primers for early detection of invasive species—A case study on the bivalve *Rangia cuneata*, currently spreading in Europe. *Mar. Environ. Res.* 2015, *112*, 48–55. [CrossRef]
- 19. Carim, K.J.; Christianson, K.R.; McKelvey, K.M.; Pate, W.M.; Silver, D.B.; Johnson, B.M.; Galloway, B.T.; Young, M.K.; Schwartz, M.K. Environmental DNA marker development with sparse biological information: A case study on opossum shrimp (*Mysis diluviana*). *PLoS ONE* **2016**, *11*, e0161664. [CrossRef]
- Dougherty, M.M.; Larson, E.R.; Renshaw, M.A.; Gantz, C.A.; Egan, S.P.; Erickson, D.M.; Lodge, D.M. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *J. Appl. Ecol.* 2016, *53*, 722–732. [CrossRef]
- 21. Forsström, T.; Vasemägi, A. Can environmental DNA (eDNA) be used for detection and monitoring of introduced crab species in the Baltic Sea? *Mar. Pollut. Bull.* **2016**, *109*, 350–355. [CrossRef]
- 22. Larson, E.R.; Renshaw, M.A.; Gantz, C.A.; Umek, J.; Chandra, S.; Lodge, D.M.; Egan, S.P. Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia* **2017**, *800*, 173–185. [CrossRef]
- 23. Piaggio, A.J.; Engeman, R.M.; Hopken, M.W.; Humphrey, J.S.; Keacher, K.L.; Bruce, W.E.; Avery, M.L. Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Mol. Ecol. Resour.* **2014**, *14*, 374–380. [CrossRef]
- 24. Davy, C.M.; Kidd, A.G.; Wilson, C.C. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLoS ONE* 2015, *10*, e0130965. [CrossRef]
- 25. Klymus, K.E.; Marshall, N.T.; Stepien, C.A. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLoS ONE* **2017**, *12*, e0177643. [CrossRef]
- Goldberg, C.S.; Sepulveda, A.; Ray, A.; Baumgardt, J.; Waits, L.P. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshw. Sci.* 2013, 32, 792–800. [CrossRef]
- 27. Xia, Z.; Zhan, A.; Gao, Y.; Zhang, L.; Haffner, G.D.; MacIsaac, H.J. Early detection of a highly invasive bivalve based on environmental DNA (eDNA). *Biol. Invasions* **2018**, *20*, 437–447. [CrossRef]
- Thomsen, P.F.; Kielgast, J.; Iversen, L.L.; Wiuf, C.; Rasmussen, M.; Gilbert, M.T.P.; Orlando, L.; Willerslev, E. Monitoring endangered freshwater biodiversity using environmental DNA. *Mol. Ecol.* 2012, 21, 2565–2573. [CrossRef] [PubMed]
- 29. Rees, H.C.; Baker, C.A.; Gardner, D.S.; Maddison, B.C.; Gough, K.C. The detection of great crested newts year round via environmental DNA analysis. *BMC Res. Notes* **2017**, *10*, 327. [CrossRef]
- Schmelzle, M.C.; Kinziger, A.P. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Mol. Ecol. Resour.* 2016, *16*, 895–908. [CrossRef] [PubMed]
- 31. Ikeda, K.; Doi, H.; Tanaka, K.; Kawai, T.; Negishi, J.N. Using environmental DNA to detect an endangered crayfish *Cambaroides japonicus* in streams. *Conserv. Genet. Resour.* **2016**, *8*, 231–234. [CrossRef]
- 32. Piggott, M.P. An environmental DNA assay for detecting Macquarie perch, *Macquaria australasica*. *Conserv. Genet. Resour.* 2017, *9*, 257–259. [CrossRef]
- 33. Brozio, S.; Manson, C.; Gourevitch, E.; Burns, T.J.; Greener, M.S.; Downie, J.R.; Hoskisson, P.A. Development and application of an eDNA method to detect the critically endangered Trinidad golden tree frog (*Phytotriades auratus*) in bromeliad Phytotelmata. *PLoS ONE* **2017**, *12*, e0170619. [CrossRef]
- 34. Laramie, M.B.; Pilliod, D.S.; Goldberg, C.S. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biol. Conserv.* **2015**, *183*, 29–37. [CrossRef]
- 35. Olson, Z.H.; Briggler, J.T.; Williams, R.N. An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildl. Res.* **2012**, *39*, 629–636. [CrossRef]

- 36. Parsons, K.M.; Everett, M.; Dahlheim, M.; Park, L. Water, water everywhere: Environmental DNA can unlock population structure in elusive marine species. *R. Soc. Open Sci.* **2018**, *5*, 180537. [CrossRef]
- Adams, C.I.M.; Knapp, M.; Gemmell, N.J.; Jeunen, G.-J.; Bunce, M.; Lamare, M.D.; Taylor, H.R. Beyond biodiversity: Can environmental DNA (eDNA) cut it as a population genetics tool? *Genes* 2019, 10, 192. [CrossRef]
- 38. Witmer, G.W. Wildlife population monitoring: Some practical considerations. *Wildl. Res.* 2005, *32*, 259. [CrossRef]
- Hoffmann, C.; Schubert, G.; Calvignac-Spencer, S. Aquatic biodiversity assessment for the lazy. *Mol. Ecol.* 2016, 25, 846–848. [CrossRef]
- 40. Jerde, C.L.; Mahon, A.R.; Chadderton, W.L.; Lodge, D.M. "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conserv. Lett.* **2011**, *4*, 150–157. [CrossRef]
- 41. Hinlo, R.; Furlan, E.; Suitor, L.; Gleeson, D. Environmental DNA monitoring and management of invasive fish: Comparison of eDNA and fyke netting. *Manag. Biol. Invasions* **2017**, *8*, 89–100. [CrossRef]
- Evans, N.T.; Shirey, P.D.; Wieringa, J.G.; Mahon, A.R.; Lamberti, G.A. Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries* 2017, 42, 90–99. [CrossRef]
- 43. Hebert, P.; Penton, E.H.; Burns, J.M.; Janzen, D.H.; Hallwachs, W. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14812–14817. [CrossRef]
- 44. Fletcher, L.M.; Zaiko, A.; Atalah, J.; Richter, I.; Dufour, C.M.; Pochon, X.; Wood, S.A.; Hopkins, G.A. Bilge water as a vector for the spread of marine pests: A morphological, metabarcoding and experimental assessment. *Biol. Invasions* **2017**, *19*, 2851–2867. [CrossRef]
- Ardura, A.; Zaiko, A.; Martinez, J.L.; Samuiloviene, A.; Borrell, Y.; Garcia-Vazquez, E. Environmental DNA evidence of transfer of North Sea molluscs across tropical waters through ballast water. *J. Molluscan Stud.* 2015, *81*, 495–501. [CrossRef]
- 46. Pielou, E.C. The measurement of diversity in different types of biological collections. *J. Theor. Biol.* **1966**, *13*, 131–144. [CrossRef]
- 47. Eichmiller, J.J.; Bajer, P.G.; Sorensen, P.W. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS ONE* **2014**, *9*, e112611. [CrossRef]
- Doi, H.; Uchii, K.; Takahara, T.; Matsuhashi, S.; Yamanaka, H.; Minamoto, T. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS ONE* 2015, *10*, e0122763. [CrossRef] [PubMed]
- 49. Uthicke, S.; Lamare, M.; Doyle, J.R. eDNA detection of corallivorous seastar (*Acanthaster cf. solaris*) outbreaks on the Great Barrier Reef using digital droplet PCR. *Coral Reefs* **2018**, *37*, 1229–1239. [CrossRef]
- Sassoubre, L.M.; Yamahara, K.M.; Gardner, L.D.; Block, B.A.; Boehm, A.B. Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environ. Sci. Technol.* 2016, 50, 10456–10464. [CrossRef] [PubMed]
- 51. Iversen, L.L.; Kielgast, J.; Sand-Jensen, K. Monitoring of animal abundance by environmental DNA—An increasingly obscure perspective: A reply to Klymus et al., 2015. *Biol. Conserv.* **2015**, *192*, 479–482. [CrossRef]
- Lacoursière-Roussel, A.; Rosabal, M.; Bernatchez, L. Estimating fish abundance and biomass from eDNA concentrations: Variability among capture methods and environmental conditions. *Mol. Ecol. Resour.* 2016, 16, 1401–1414. [CrossRef] [PubMed]
- Bista, I.; Carvalho, G.R.; Tang, M.; Walsh, K.; Zhou, X.; Hajibabaei, M.; Shokralla, S.; Seymour, M.; Bradley, D.; Liu, S.; et al. Performance of amplicon and shotgun sequencing for accurate biomass estimation in invertebrate community samples. *Mol. Ecol. Resour.* 2018, *18*, 1020–1034. [CrossRef] [PubMed]
- Klymus, K.E.; Richter, C.A.; Chapman, D.C.; Paukert, C. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biol. Conserv.* 2015, 183, 77–84. [CrossRef]
- 55. Stewart, K.A. Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. *Biodivers. Conserv.* **2019**, *28*, 1–19. [CrossRef]
- 56. Seymour, M.; Durance, I.; Cosby, B.J.; Ransom-Jones, E.; Deiner, K.; Ormerod, S.J.; Colbourne, J.K.; Wilgar, G.; Carvalho, G.R.; de Bruyn, M.; et al. Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. *Commun. Biol.* **2018**, *1*, 4. [CrossRef]

- 57. Collins, R.A.; Wangensteen, O.S.; O'Gorman, E.J.; Mariani, S.; Sims, D.W.; Genner, M.J. Persistence of environmental DNA in marine systems. *Commun. Biol.* **2018**, *1*, 185. [CrossRef]
- 58. Takahara, T.; Minamoto, T.; Doi, H. Effects of sample processing on the detection rate of environmental DNA from the Common Carp (*Cyprinus carpio*). *Biol. Conserv.* **2015**, *183*, 64–69. [CrossRef]
- 59. Wilcox, T.M.; McKelvey, K.S.; Young, M.K.; Sepulveda, A.J.; Shepard, B.B.; Jane, S.F.; Whiteley, A.R.; Lowe, W.H.; Schwartz, M.K. Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis*. *Biol. Conserv.* **2016**, *194*, 209–216. [CrossRef]
- 60. Darling, J.A.; Mahon, A.R. From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environ. Res.* **2011**, *111*, 978–988. [CrossRef] [PubMed]
- 61. Ficetola, G.F.; Pansu, J.; Bonin, A.; Coissac, E.; Giguet-Covex, C.; De Barba, M.; Gielly, L.; Lopes, C.M.; Boyer, F.; Pompanon, F.; et al. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Mol. Ecol. Resour.* **2015**, *15*, 543–556. [CrossRef] [PubMed]
- 62. Lahoz-Monfort, J.J.; Guillera-Arroita, G.; Tingley, R. Statistical approaches to account for false-positive errors in environmental DNA samples. *Mol. Ecol. Resour.* **2016**, *16*, 673–685. [CrossRef]
- 63. Muha, T.P.; Rodríguez-Rey, M.; Rolla, M.; Tricarico, E. Using Environmental DNA to improve species distribution models for freshwater invaders. *Front. Ecol. Evol.* **2017**, *5*, 158. [CrossRef]
- Willoughby, J.R.; Wijayawardena, B.K.; Sundaram, M.; Swihart, R.K.; DeWoody, J.A. The importance of including imperfect detection models in eDNA experimental design. *Mol. Ecol. Resour.* 2016, 16, 837–844. [CrossRef]
- 65. Mackenzie, D.I.; Nichols, J.D.; Hines, J.E.; Knutson, M.G.; Franklin, A.B.; Knu, M.G.; Franklin, A.B. Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly. *Ecology* **2003**, *84*, 2200–2207. [CrossRef]
- 66. Hunter, M.E.; Oyler-McCance, S.J.; Dorazio, R.M.; Fike, J.A.; Smith, B.J.; Hunter, C.T.; Reed, R.N.; Hart, K.M. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS ONE* **2015**, *10*, e0121655. [CrossRef] [PubMed]
- Böhm, M.; Collen, B.; Baillie, J.E.M.; Bowles, P.; Chanson, J.; Cox, N.; Hammerson, G.; Hoffmann, M.; Livingstone, S.R.; et al. The conservation status of the world's reptiles. *Biol. Conserv.* 2013, 157, 372–385. [CrossRef]
- Van Dijk, P.P.; Iverson, J.; Rhodin, A.; Shaffer, B.; Bour, R. Turtles of the World, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. *Chelonian Res. Monogr.* 2014, 5, 329–479. [CrossRef]
- 69. Egeter, B.; Peixoto, S.; Brito, J.C.; Jarman, S.; Puppo, P.; Velo-Antón, G. Challenges for assessing vertebrate diversity in turbid Saharan water-bodies using environmental DNA. *Genome* **2018**, *61*, 807–814. [CrossRef]
- Lacoursière-Roussel, A.; Dubois, Y.; Normandeau, E.; Bernatchez, L.; Adamowicz, S. Improving herpetological surveys in eastern North America using the environmental DNA method. *Genome* 2016, 59, 991–1007. [CrossRef]
- 71. Hart, K.M.; Cherkiss, M.S.; Smith, B.J.; Mazzotti, F.J.; Fujisaki, I.; Snow, R.W.; Dorcas, M.E. Home range, habitat use, and movement patterns of non-native Burmese pythons in Everglades National Park, Florida, USA. *Anim. Biotelemetry* **2015**, *3*, 8. [CrossRef]
- 72. Kucherenko, A.; Herman, J.E.; III, E.M.E.; Urakawa, H. Terrestrial snake environmental DNA accumulation and degradation dynamics and its environmental application. *Herpetologica* **2018**, *74*, 38–49. [CrossRef]
- Baker, S.J.; Niemiller, M.L.; Stites, A.J.; Ash, K.T.; Davis, M.A.; Dreslik, M.J.; Phillips, C.A. Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces ophiodiicola* in crayfish burrows. *Conserv. Genet. Resour.* 2018, 1–3. [CrossRef]
- 74. Halstead, B.J.; Wood, D.A.; Bowen, L.; Waters, S.C.; Vandergast, A.G.; Ersan, J.S.; Skalos, S.M.; Casazza, M.L. *An Evaluation of the Efficacy of Using Environmental DNA (eDNA) to Detect Giant Gartersnakes (Thamnophis gigas);* USGS Open-File Reports; U.S. Geological Survey: Reston, VA, USA, 2017; 41p.
- 75. Halstead, B.J.; Wylie, G.D.; Casazza, M.L. Habitat suitability and conservation of the Giant Gartersnake (*Thamnophis gigas*) in the Sacramento Valley of California. *Copeia* **2010**, 2010, 591–599. [CrossRef]
- Cannon, M.V.; Hester, J.; Shalkhauser, A.; Chan, E.R.; Logue, K.; Small, S.T.; Serre, D. In silico assessment of primers for eDNA studies using PrimerTree and application to characterize the biodiversity surrounding the Cuyahoga River. *Sci. Rep.* 2016, *6*, 22908. [CrossRef]

- 77. de Souza, L.S.; Godwin, J.C.; Renshaw, M.A.; Larson, E. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS ONE* **2016**, *11*, e0165273. [CrossRef]
- 78. Feist, S.M.; Jones, R.L.; Copley, J.L.; Pearson, L.S.; Berry, G.A.; Qualls, C.P. Development and validation of an environmental DNA method for detection of the Alligator Snapping Turtle (*Macrochelys temminckii*). *Chelonian Conserv. Biol.* 2018, 17, 271–279. [CrossRef]
- 79. Kelly, R.P.; Port, J.A.; Yamahara, K.M.; Crowder, L.B. Using environmental DNA to census marine fishes in a large mesocosm. *PLoS ONE* **2014**, *9*, e86175. [CrossRef]
- 80. Kundu, S.; Kumar, V.; Tyagi, K.; Chandra, K. Environmental DNA (eDNA) testing for detection of freshwater turtles in a temple pond. *Herpetol. Notes* **2018**, *11*, 369–371.
- 81. Raemy, M.; Ursenbacher, S. Detection of the European pond turtle (*Emys orbicularis*) by environmental DNA: Is eDNA adequate for reptiles? *Amphibia-Reptilia* **2018**, *39*, 135–143. [CrossRef]
- Wilson, J.-J.; Sing, K.-W.; Chen, P.-N.; Zieritz, A. Tracking the southern river terrapin (*Batagur affinis*) through environmental DNA: Prospects and challenges. *Mitochondrial DNA Part A* 2017, 29, 862–866. [CrossRef] [PubMed]
- 83. Rodrigues, J.F.M.; Coelho, M.T.P.; Varela, S.; Diniz-Filho, J.A.F. Invasion risk of the pond slider turtle is underestimated when niche expansion occurs. *Freshw. Biol.* **2016**, *61*, 1119–1127. [CrossRef]
- 84. Fonseca, V.G. Pitfalls in relative abundance estimation using eDNA metabarcoding. *Mol. Ecol. Resour.* 2018, 18, 923–926. [CrossRef]
- 85. Ernst, C.H.; Lovich, J.E. *Turtles of the United States and Canada*, 2nd ed.; JHU Press: Baltimore, MD, USA, 2009; ISBN 0801891213.
- 86. Iverson, J.B. Biomass in turtle populations: A neglected subject. Oecologia 1982, 55, 69–76. [CrossRef]
- 87. Congdon, J.D.; Greene, J.L.; Gibbons, J.W. Biomass of freshwater turtles: A geographic comparison. *Am. Midl. Nat.* **1986**, *115*, 165–173. [CrossRef]
- 88. Ernst, C.H. Population dynamics and activity cycles of *Chrysemys picta* in southeastern Pennsylvania. *J. Herpetol.* **1971**, *5*, 151–160. [CrossRef]
- 89. Dunker, K.J.; Sepulveda, A.J.; Massengill, R.L.; Olsen, J.B.; Russ, O.L.; Wenburg, J.K.; Antonovich, A. Potential of Environmental DNA to Evaluate Northern Pike (*Esox lucius*) Eradication Efforts: An Experimental Test and Case Study. *PLoS ONE* **2016**, *11*, e0162277. [CrossRef] [PubMed]
- 90. Tsuji, S.; Yamanaka, H.; Minamoto, T. Effects of water pH and proteinase K treatment on the yield of environmental DNA from water samples. *Limnology* **2017**, *18*, 1–7. [CrossRef]
- 91. Deiner, K.; Walser, J.C.; Mächler, E.; Altermatt, F. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biol. Conserv.* **2015**, *183*, 53–63. [CrossRef]
- 92. Taberlet, P.; Griffin, S.; Goossens, B.; Questiau, S.; Manceau, V.; Escaravage, N.; Waits, L.P.; Bouvet, J. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res.* **1996**, *24*, 3189–3194. [CrossRef]
- Olds, B.P.; Jerde, C.L.; Renshaw, M.A.; Li, Y.; Evans, N.T.; Turner, C.R.; Deiner, K.; Mahon, A.R.; Brueseke, M.A.; Shirey, P.D.; et al. Estimating species richness using environmental DNA. *Ecol. Evol.* 2016, *6*, 4214–4226. [CrossRef]
- 94. Sow, F.B.; Gallup, J.M.; Sacco, R.E.; Ackermann, M.R. Laser capture microdissection revisited as a tool for transcriptomic analysis: Application of an excel-based qPCR preparation software (PREXCEL-Q). *Int. J. Biomed. Sci.* **2009**, *5*, 105–124. [PubMed]
- 95. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef]
- McKee, A.M.; Spear, S.F.; Pierson, T.W. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biol. Conserv.* 2015, 183, 70–76. [CrossRef]
- 97. Jonckheere, A.R. A distribution-free k-sample test against ordered alternatives. *Biometrika* **1954**, *41*, 133. [CrossRef]
- 98. R Core Team. *R: A Language and Environment for Statistical Computing;* R Core Team: Vienna, Austria, 2013. Available online: http://www.R-project.org/ (accessed on 24 February 2019).

- Mächler, E.; Deiner, K.; Spahn, F.; Altermatt, F. Fishing in the water: Effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. *Environ. Sci. Technol.* 2016, 50, 305–312. [CrossRef]
- Furlan, E.M.; Gleeson, D.; Hardy, C.M.; Duncan, R.P. A framework for estimating the sensitivity of eDNA surveys. *Mol. Ecol. Resour.* 2016, 16, 641–654. [CrossRef] [PubMed]
- Nathan, L.M.; Simmons, M.; Wegleitner, B.J.; Jerde, C.L.; Mahon, A.R. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. *Environ. Sci. Technol.* 2014, 48, 12800–12806. [CrossRef] [PubMed]
- Yang, R.; Paparini, A.; Monis, P.; Ryan, U. Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium oocysts* in faecal samples. *Int. J. Parasitol.* 2014, 44, 1105–1113. [CrossRef]
- 103. Rački, N.; Dreo, T.; Gutierrez-Aguirre, I.; Blejec, A.; Ravnikar, M. Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. *Plant Methods* 2014, 10, 42. [CrossRef] [PubMed]
- 104. Barnes, M.A.; Turner, C.R.; Jerde, C.L.; Renshaw, M.A.; Chadderton, W.L.; Lodge, D.M. Environmental conditions influence eDNA persistence in aquatic systems. *Environ. Sci. Technol.* 2014, 48, 1819–1827. [CrossRef] [PubMed]
- 105. Renshaw, M.A.; Olds, B.P.; Jerde, C.L.; Mcveigh, M.M.; Lodge, D.M. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. *Mol. Ecol. Resour.* 2015, *15*, 168–176. [CrossRef]
- 106. Wegleitner, B.J.; Jerde, C.L.; Tucker, A.; Chadderton, W.L.; Mahon, A.R. Long duration, room temperature preservation of filtered eDNA samples. *Conserv. Genet. Resour.* **2015**, *7*, 789–791. [CrossRef]
- 107. Pierson, T.W.; McKee, A.M.; Spear, S.F.; Maerz, J.C.; Camp, C.D.; Glenn, T.C. Detection of an enigmatic Plethodontid salamander using environmental DNA. *Copeia* **2016**, *104*, 78–82. [CrossRef]
- 108. Wilson, I.G. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **1997**, *63*, 3741–3751.
- Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR inhibitors—Occurrence, properties and removal. J. Appl. Microbiol. 2012, 113, 1014–1026. [CrossRef]
- Li, J.; Lawson Handley, L.-J.; Read, D.S.; Hänfling, B. The effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding. *Mol. Ecol. Resour.* 2018, *18*, 1102–1114. [CrossRef]
- 111. Weldon, P.J.; Demeter, B.J.; Rosscoe, R. A survey of shed skin-eating (Dermatophagy) in amphibians and reptiles. *J. Herpetol.* **1993**, *27*, 219. [CrossRef]
- 112. Ernst, C.H. Growth of the painted turtle, *Chrysemys picta*, in Southeastern Pennsylvania. *Herpetologica* **1971**, 27, 135–141.
- 113. Parmenter, R.R. Digestive turnover rates in freshwater turtles: The influence of temperature and body size. *Comp. Biochem. Physiol. Part A Physiol.* **1981**, *70*, 235–238. [CrossRef]
- Northmore, D.; Granda, A. Ocular dimensions and schematic eyes of freshwater and sea turtles. *Vis. Neurosci.* 1991, 7, 627–635. [CrossRef] [PubMed]
- 115. Sansom, B.J.; Sassoubre, L.M. Environmental DNA (eDNA) Shedding and Decay Rates to Model Freshwater Mussel eDNA Transport in a River. *Environ. Sci. Technol.* 2017, *51*, 14244–14253. [CrossRef] [PubMed]
- 116. Goldberg, C.S.; Turner, C.R.; Deiner, K.; Klymus, K.E.; Thomsen, P.F.; Murphy, M.A.; Spear, S.F.; McKee, A.; Oyler-McCance, S.J.; Cornman, R.S.; et al. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods Ecol. Evol.* **2016**, *7*, 1299–1307. [CrossRef]
- 117. Taberlet, P.; Bonin, A.; Zinger, L.; Coissac, E. *Environmental DNA: For Biodiversity Research and Monitoring;* Oxford University Press: Oxford, UK, 2018; ISBN 0198767226.
- 118. Deiner, K.; Bik, H.M.; Mächler, E.; Seymour, M.; Lacoursière-Roussel, A.; Altermatt, F.; Creer, S.; Bista, I.; Lodge, D.M.; de Vere, N.; et al. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Mol. Ecol.* 2017, 26, 5872–5895. [CrossRef] [PubMed]
- 119. Takahashi, M.K.; Meyer, M.J.; Mcphee, C.; Gaston, J.R.; Venesky, M.D.; Case, B.F. Seasonal and diel signature of eastern hellbender environmental DNA. *J. Wildl. Manag.* **2018**, *82*, 217–225. [CrossRef]

- Spear, S.F.; Groves, J.D.; Williams, L.A.; Waits, L.P. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biol. Conserv.* 2015, 183, 38–45. [CrossRef]
- 121. O'Donnell, J.L.; Kelly, R.P.; Shelton, A.O.; Samhouri, J.F.; Lowell, N.C.; Williams, G.D. Spatial distribution of environmental DNA in a nearshore marine habitat. *PeerJ* **2017**, *5*, e3044. [CrossRef]
- 122. Deiner, K.; Altermatt, F. Transport distance of invertebrate environmental DNA in a natural river. *PLoS ONE* **2014**, *9*, e88786. [CrossRef]
- 123. Jane, S.F.; Wilcox, T.M.; Mckelvey, K.S.; Young, M.K.; Schwartz, M.K.; Lowe, W.H.; Letcher, B.H.; Whiteley, A.R. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Mol. Ecol. Resour.* 2015, 15, 216–227. [CrossRef]
- 124. Nukazawa, K.; Hamasuna, Y.; Suzuki, Y. Simulating the advection and degradation of the environmental DNA of common carp along a river. *Environ. Sci. Technol.* **2018**, *52*, 10562–10570. [CrossRef]
- 125. Wilson, C.C.; Wozney, K.M.; Smith, C.M. Recognizing false positives: Synthetic oligonucleotide controls for environmental DNA surveillance. *Methods Ecol. Evol.* **2015**, *7*, 23–29. [CrossRef]
- 126. Wilcox, T.M.; McKelvey, K.S.; Young, M.K.; Jane, S.F.; Lowe, W.H.; Whiteley, A.R.; Schwartz, M.K. Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS ONE* 2013, *8*, e59520. [CrossRef] [PubMed]
- 127. Turner, C.R.; Barnes, M.A.; Xu, C.C.Y.; Jones, S.E.; Jerde, C.L.; Lodge, D.M. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods Ecol. Evol.* **2014**, *5*, 676–684. [CrossRef]
- 128. Hinlo, R.; Gleeson, D.; Lintermans, M.; Furlan, E. Methods to maximise recovery of environmental DNA from water samples. *PLoS ONE* **2017**, *12*, e0179251. [CrossRef]
- 129. Williams, K.E.; Huyvaert, K.P.; Piaggio, A.J. Clearing muddled waters: Capture of environmental DNA from turbid waters. *PLoS ONE* 2017, 12, e0179282. [CrossRef]
- Beans, C. Core Concept: Environmental DNA helps researchers track pythons and other stealthy creatures. *Proc. Natl. Acad. Sci. USA* 2018, 115, 8843–8845. [CrossRef] [PubMed]
- Levi, T.; Allen, J.M.; Bell, D.; Joyce, J.; Russell, J.R.; Tallmon, D.A.; Vulstek, S.C.; Yang, C.; Yu, D.W. Environmental DNA for the enumeration and management of Pacific salmon. *Mol. Ecol. Resour.* 2018, 394445. [CrossRef] [PubMed]
- Rees, H.C.; Bishop, K.; Middleditch, D.J.; Patmore, J.R.M.; Maddison, B.C.; Gough, K.C. The application of eDNA for monitoring of the great crested newt in the UK. *Ecol. Evol.* 2014, *4*, 4023–4032. [CrossRef] [PubMed]



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).