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Research Article

Tracing the quagga mussel invasion along the Rhine river system using eDNA markers: early detection and surveillance of invasive zebra and quagga mussels

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Abstract

Early detection and monitoring of invasive species is important for the development of effective measures directed at minimising the negative effects of invaders. In the early stages of invasion, aquatic invasive species are typically rare and their detection using costly and time-consuming field surveys is often challenging. Environmental DNA (eDNA) methods are increasingly applied in freshwater systems to detect and quantify target species with relative ease over large geographic scales and across the invasion fronts. In this study we test eDNA detection and quantification methods for invasive zebra and quagga mussels. Both mussel species have invaded widely in North America and Europe and show strong negative ecosystem-wide impacts. We extracted DNA from filtered water samples which we collected along the Rhine catchment in Switzerland, Germany and The Netherlands, including the known invasion area of the zebra mussel and the invasion front of the quagga mussel. Standard PCR and quantitative PCR (qPCR) methods were compared for detection and qPCR was used to quantify the eDNA signal for each species. Our results show that the invasion front of the quagga mussel has moved southwards, including areas where this species had not been detected previously with traditional benthic invertebrate sampling methods. Standard PCR and qPCR showed a similar performance in detecting both mussel species. Moreover, the eDNA quantification of the two species showed low variance within sampling site and matched with expected densities of zebra and quagga mussels based on previous field survey studies. The tested eDNA methods are cost effective and have the potential to be widely applied for the surveillance of zebra and quagga mussels in the future.

Key words: eDNA, targeted species detection, freshwater, *Dreissena polymorpha, Dreissena rostriformis bugensis*, invasive species, Cytochrome c oxidase I, qPCR

Introduction

Invasive species have strong negative impacts on biodiversity and cause high economic costs in freshwater systems worldwide (Sala et al. 2000). Early detection and surveillance of invasive species is important in order to plan measures to slow down their spread and to mitigate their effects. Nevertheless, early detection and quantification of aquatic invasive species, e.g. by kicknet sampling or scuba diving is often difficult, laborious and potentially inaccurate (Barbour et al. 1999; Stucki 2010), in particular for small freshwater invertebrates, which often have patchy distribution patterns (Arscott et al. 2003). The detection of non-native species with the potential to become invasive can be particularly difficult where densities are low. This is often the case during the early lag-phase of establishment (Lockwood et al. 2007), or when primary habitats are particularly inaccessible for surveys, e.g. in deeper lakes. In such cases, the detection and quantification of species from environmental DNA (eDNA) extracted from water samples may have several advantages over traditional surveillance

methods, as has been demonstrated for the American bullfrog (Dejean et al. 2012), the Asian carp (Jerde et al. 2013), the invasive New Zealand mudsnail (Goldberg et al. 2013) or the invasive rusty crayfish (Dougherty et al. 2016).

The major advantage of the eDNA method is that the target organisms do not need to be found and identified. Instead it only requires collection of water samples, concentrating the organic material and extracting the eDNA, e.g. from filter papers. eDNA comprises extracellular and cell-bound DNA which organisms release as a by-product of excretion or the shedding of cells (e.g. in hair or skin) into the water column (Thomsen and Willerslev 2015). The occurrence of target organisms in the eDNA samples can then be detected by end-point Polymerase Chain Reaction (standard PCR) with species specific primers (Goldberg et al. 2013; Mächler et al. 2014). Particularly when applied to one or a few species of interest, eDNA approaches coupled with species or lineage-specific PCR may allow assessment of species occurrence in high temporal and spatial resolution. Further, application of standardized sampling and molecular protocols may allow comparisons across studies and surveillance programs (Thomsen and Willerslev 2015).

Quantitative PCR (qPCR) with species specific primers allows the quantification of target DNA in eDNA samples and was shown to be more sensitive to lower copy numbers than standard PCR (Wilcox et al. 2013). Such quantitative eDNA estimates may be used as a proxy for population densities at a specific location. Several researchers have successfully correlated eDNA concentrations with densities or biomass of target freshwater organisms in captivity, e.g. for fish (Takahara et al. 2012), amphibians (Thomsen et al. 2012) or New Zealand mudsnails (Goldberg et al. 2013). For two amphibian species, density estimates from field survey data have shown good positive correlation with eDNA estimates of population size (Pilliod et al. 2013). However, only very few studies have investigated the potential of standard PCR and qPCR for early detection and quantification of invasive species over wide geographic scales and few have assessed the potential of eDNA quantification for more than one species within the same freshwater system and compared the results to field survey data.

The zebra mussel (*Dreissena polymorpha* Pallas, 1771) and the quagga mussel (*Dreissena rostriformis bugensis* Andrusov, 1897) are two closely related species originating from the Ponto-Caspian region. Both species are invasive in North America and Europe (Mills et al. 1996; Therriault et al. 2005; Zhulidov et al. 2010), with strong negative impacts on the ecology of the invaded water bodies (Vanderploeg et al. 2002; Strayer 2009; Higgins and Vander Zanden 2010) and economics (Pimentel et al. 2005). The two species share a similar life cycle, both produce pseudofaeces and exhibit similarly high filtration rates (Ackerman et al. 1994; Diggins 2000), and may thus also show comparably high eDNA shedding rates. Since the 19th century the zebra mussel has colonized many large rivers and navigable lakes in Western Europe, but started colonizing Swiss water bodies much later in the 1960s. The quagga mussel only arrived around 2004 in the Netherlands and in the Rhine-Main-Danube channel (Imo et al. 2010; Heiler et al. 2013) and is currently spreading southwards along the Rhine system (Matthews et al. 2014). In Switzerland, the quagga mussel had not been detected before this study. The quagga mussel was found to cope better with low temperatures (Roe and MacIsaac 1997) and lower nutrient levels (Baldwin et al. 2002) than the zebra mussel and may thus colonize colder or more oligotrophic water bodies, for example in higher altitudes. They may also colonize lentic systems to greater depths, potentially attaching to surfaces and clogging water intake pipes of drinking water plants.

In order to plan measures against the further spread of quagga mussels and for the mitigation of expected negative impacts, it is important to monitor the spread of this species. We therefore examined if eDNA methods using standard PCR and qPCR with species specific primers provide an efficient and cost effective method for the surveillance of zebra and quagga mussels. We collected water samples along the River Rhine system, from Lake Constance to the Lower Rhine in the Netherlands (Figure 1). While quagga mussels had previously not been detected upstream of Kehl (Kinzelbach 1992; Bij de Vaate et al. 2002; Heiler et al. 2013), zebra mussels were known to be present at all sampling sites, thus serving as a positive control for the species specific eDNA detection. As eDNA is washed downstream, the samples may represent the upstream community up to several kilometers upstream of the sampling site (Deiner and Altermatt 2014). We chose to use filtration and eDNA extraction methods previously used by Deiner et al. (Deiner et al. 2015) who filtered and extracted DNA from water samples in a dedicated DNA-free facility in the laboratory. In addition, we also filtered water samples directly in the field, in order to find out whether this simpler approach was free of cross-contamination between sampling sites. Applying standard PCR and qPCR using the species specific primers published by (Bronnenhuber and Wilson 2013) we addressed the following points:

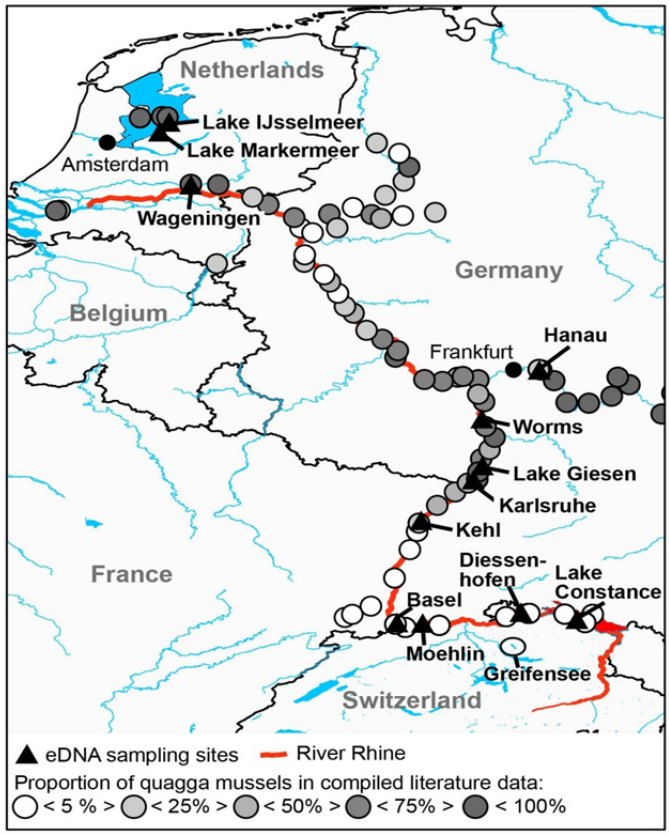

Figure 1. Sampling sites (black triangles, for coordinates see Supplementary material Table S1), where eDNA samples were collected along the River Rhine catchment. Mussels were collected from Lake IJsselmeer, Lake Markermeer Hanau and Greifensee for tissue extracted DNA. Circles with different shades of grey indicate the proportion of quagga mussel density (individuals per m^2) in relation to the total dreissenid density (zebra plus quagga mussels). These density estimates originate from field survey data, which we compiled from the literature. Most field density estimates for the Rivers Rhine and Main were collected in 2009 by Heiler et al. (2013), those for the Swiss River Rhine and Lake Constance by John Hesselschwerdt and Jutta Mürle in 2014 (Hesselschwerdt et al. 2014), those for Lake Markermeer and Lake IJsselmeer in 2011 by Matthews et al. (2014) and in 2012 by Heiler et al. (2013), and those from the Lower Rhine in Wageningen where collected in 2011 by Matthews et al. (2015) and Leuven et al. (2014).

1. We tested the detection of eDNA of zebra and quagga mussels in field filtered and lab filtered water samples using standard PCR. In particular, we were interested in whether the quagga mussel can be detected upstream of Kehl, where it has not been reported so far.
2. We estimated the lowest concentration of target DNA of zebra and quagga mussels that can still be detected in eDNA samples with EvaGreen qPCR method.
3. We quantified and compared the concentrations of target eDNA of zebra and quagga mussel with qPCR.
4. Finally, we discuss the potential application of eDNA detection and quantification with standard PCR and qPCR for the surveillance of invasive zebra and quagga mussels.

Methods

Field sampling

Environmental DNA (eDNA) samples were collected at twelve sites in the River Rhine catchment in July and August 2014 (Figure 1, Table 1, Supplementary material Table S1). At each site, three water samples of 1 L volume were collected from the shore (water depth approx. 1 m) with a clean 10 L bucket, which was rinsed five times few meters downstream of the actual sampling site before the sample was taken. Each sample was filtered directly in the field on a glass fibre filter (GF/F Glass fibre filters, 25 mm diameter, 0.7 µm average pore size) using a clean filter holder (GE Healthcare, Whatman) and a disposable 50 mL syringe. In order to prevent contamination of filters with non-site specific eDNA, filters were only touched with clean forceps and filter holder and syringes only with new disposable gloves. Filter holders, and forceps were bleached (10% bleach solution) and treated with UV-light for 20 minutes, while filters were also treated with UV-light before use. This procedure used to be the standard for cleaning eDNA equipment, while 50% bleach is recommended in a more recent paper by Goldberg et al. (2016). For the filtration of each 1 L sample we needed between one and four filters, depending on the amount of organic and inorganic material present in the water. Filters were placed into fresh 1.5 mL Eppendorf tubes, frozen immediately in

Table 1. eDNA sampling sites with geographic information, sampling date, filtration method, indication whether field- or lab-filter controls were analyzed, expected quagga mussel presence and sample ID. The filtration method indicates where the samples were filtered. We also show if we expected to find quagga mussels. The names in bold letter indicate how the samples are named throughout the paper.

Place	Water body	Sampling date	Field filtered	Field filter control	Lab filtered	Lab filter control	Quagga mussels expetect	ID
Altnau	**Lake Constance**	01.07.2014	Yes	Yes	Yes	Yes	No	BS
Diessenhofen	Rhine River	01.07.2014	Yes	No	Yes	Yes	No	DH
Moehlin	Rhine River	03.07.2014	Yes	Yes	Yes	No	No	Mö
Basel (harbor)	Rhine River	03.07.2014	Yes	Yes	Yes	Yes	No	Ba
Kehl (harbor)	Rhine River	11.07.2014	Yes	Yes	Yes	Yes	Yes	Ke
Karlsruhe (harbor)	Rhine River	10.07.2014	Yes	Yes	Yes	Yes	Yes	Ka
Dettenheim	**Lake Giesen**	10.07.2014	Yes	Yes	Yes	Yes	Yes	De
Worms	Rhine River	09.07.2014	Yes	Yes	Yes	Yes	Yes	Wo
Hanau	Main River	09.07.2014	Yes	Yes	Yes	Yes	Yes	Ha
Wageningen	Rhine River	12.08.2014	Yes	Yes	No	No	Yes	Wa
Lelystad	**IJsselmeer**	11.08.2014	Yes	Yes	No	No	Yes	IJ
Almere	**Markermeer**	11.08.2014	Yes	No	No	No	Yes	Ma

a dewar vessel and kept at −80°C until DNA was extracted. As negative field controls we brought 1 L of UV-treated DNA-free water to each site and filtered it according to the above procedure. For nine sampling sites we also collected water samples by submerging a 1 L octagonal PET bottle (VWR International, Radnor, PA, USA) with a gloved hand just below the surface near the shore. The water samples were transported in an ice filled cooling box and filtered within 36 hours in a laminar flow hood in a DNA-clean facility the same way as described above for the field filtered samples. All bottles were previously rinsed with 10% bleach, rinsed well with water and pre-decontaminated by a 20 minute UV-light treatment and sealed before use. As negative lab controls, we transported 1 L of UV treated DNA-free water to each of the field sites, where it was filled into an octagonal PET bottle and subsequently treated like the lab filtered samples.

eDNA extraction protocols:

For targeted detection of zebra and quagga mussels, we extracted eDNA from the filters using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) with some modifications as described in Deiner et al. (2015), except for the following steps: After lysis we shook the content in the closed upside down Eppendorf tube towards the lid, and with a sharp pointed needle punched a hole into the bottom of the tube. A new needle was used for each sample. Subsequently each tube was inserted into a second 1.5 ml Eppendorf tube and centrifuged for 3 min at 6000 g. The extraction was continued with the flow-through, while the upper tube containing the dry filter was discarded. This procedure was chosen in order to prevent cross-contamination between samples and loss of sample solution during the removal of the filter. The extractions were performed in a laminar flow hood in a dedicated DNA-clean facility as described by Fulton (2012) and Deiner et al. (2015) and all equipment including pipettes and needles were treated with UV-light for 20 minutes before use. For the twelve negative extraction controls we used clean, UV-treated filters. For those samples for which we used more than one filter (per 1 L sample), we pooled equal amounts of extract from each filter. For the eDNA quantification with qPCR, we corrected the eDNA concentration estimates for higher total elution volumes of pooled samples. In 14 test extractions we measured eDNA concentrations between 0.9 and 6.4 ng/µl with Qubit 2.0 dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). All DNA extracts were kept at 4 °C until further use.

Species specific primers:

We used the species specific primers published by Bronnenhuber and Wilson (2013) for PCR amplification, targeting COI gene in mitochondrial DNA (Table 2). For species detection we used the DbuCOI3 primer pair, amplifying a fragment of 164 bp of the quagga mussel COI-sequence, and DpoCOI3 primer pair amplifying a 254 bp fragment of the zebra mussel COI-sequence. We tested both primer pairs for species specificity in PCR's using DNA from the tissue of four zebra and four quagga mussels (zebra mussel tissue originating from Lake Greifensee (2×), Lake IJsselmeer and Lake Markermeer, and quagga mussel tissue originating from River Main (2×), Lake IJsselmeer and Lake Markermeer). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen GmbH,

Table 2. Primers used for detection of zebra and quagga mussels (Bronnenhuber and Wilson 2013). We show the primer sequences, and the mismatches with the primer sequence at the same locus of the congener species (either zebra or quagga mussel) are shown in bold font. Further, we present the number of mismatches in the primer sequence (MMs), the length of the amplified fragment, the annealing temperature used in the PCR protocols (TA), the estimated melting temperature (TM) and GC-content of the primer sequences (% GC) and whether cross amplification (cross amp.) with the non-target species (either zebra or quagga mussels, respectively) was detected by Bronnenhuber and Wilson (2013) or by us.

Target species	Primer	Primer sequence	MMs	Fragment length	TA	TM	% GC	Cross amp.
Quagga mussel	DbuCOI3F	G**G**GGTTGAAC**A**TTATAYCC**A**CC**G**TT	4	164	66	57	48	No
	DbuCOI3R	AAA**C**TGATGACA**C**CC**G**GCACG	3			57.7	57	
Zebra mussel	DpoCOI3F	GCT**A**AG**G**GC**A**CCTGGAAG**C**GT	4	254	66	59	61	No
	DpoCOI3R	C**A**CC**C**CC**G**AATCC**T**CC**T**TC**C**CT	6			59.3	63	

Hilden, Germany), following the manufacturer's protocol and PCRs performed and products visualized as described below. We also blasted each of the primer sequences against the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/genbank/) testing for potential amplification of non-target sequences of other aquatic species.

Detection of target species by standard PCR:

In order to detect the presence of zebra and quagga mussel DNA, we amplified target sequences with PCR, multiplexing DpoCOI3 and DbuCOI3 primers and visualized the products on an agarose gel. We tested all samples filtered in the field and in the laboratory and included four types of negative controls (Supplementary material Table S2): field filtered negative controls (N=10), lab filtered negative controls (N=8), extraction negative controls (N=14) and PCR controls (N=12) containing only UV-light treated nuclease-free water (Sigma). PCR's of each sample were run in triplicate. If not all of the three triplicates of a sample were either only positive or only negative for the PCR-amplification of the target species, the PCR was again repeated in triplicate for this ambiguous sample in order to exclude a false positive or false negative result. For PCRs on eDNA and tissue extracted DNA we used Multiplex PCR Master Mix (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The final concentrations of forward and reverse primers were 0.2 µM and we used 2 µL of extracted eDNA per 15 µL reaction volume. The thermal cycling regime was 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 66 °C for 90 s and 72 °C for 90 s. A final extension step of 72 °C for 10 min was carried out and the PCR product was stored at 4 °C until further analysis. We confirmed the resulting PCR products on a 1.4% agarose gel stained with PeqGreen (Peqlab, Erlanden. Germany) and compared them to a 100bp ladder (Promega, Madison, WI, USA).

Quantifying target species by quantitative PCR:

Quantitative PCR (qPCR) reactions were run in triplicates on a LightCycler 480 Real-Time PCR System (Hoffmann-La Roche Ltd) in 15 µL reaction volumes using the same protocol and reagent concentrations as described for the PCR above, except that we added 0.75 µL Evagreen to each reaction and run separate singleplex tests for zebra and quagga mussel primers. For each of the pooled samples, the eDNA concentration was diluted two times and 3 µL of the dilute was used in the qPCR reaction (for exceptions see Supplementary material Table S2) in order to have enough volume for all qPCR replicates. We tested only lab filtered samples except for the sites Wageningen, Lake IJsselmeer and Lake Markermeer where only field filtered samples were available. We also included seven filtration negative controls, five extraction controls and one PCR control containing only UV-light treated nuclease-free water.

As qPCR standards we amplified PCR product from tissue extracted DNA for each primer pair (DbuCOI3 and DpoCOI3). Each PCR product was then purified using the centrifugation protocol of the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), eluted with 120 µL nuclease-free water and the concentration of the elute measured with Qubit. qPCR standards were subsequently prepared from the purified PCR products in a 10× dilution series for each target species (Figure 3) in DNA-low binding tubes (Eppendorf AG, Hamburg, Germany), kept at 4 °C and used in qPCR within 48 hours. The DNA concentrations were measured for the two highest concentrated standards of each dilution series with Qubit and calculated for all other standard dilutions. All samples, negative controls and standards were run on the same qPCR plate. Zebra mussel standards containing between 1.1 and 1.1×10^{10} amplicons per µL and quagga mussel standards containing between 0.8 and 8×10^{9} ampli-

cons per µL were included in six replicates. Amplification curves, Cq values, melting temperatures and melting curves were analysed in the Light Cycler 480 Software, Version 1.5 (Roche Diagnostics). We calculated the number of target sequence copies per µL eDNA sample as the concentration extrapolated from the standard curve (ng/µL) divided by the dilution factor (Supplementary material Table S2) and converted the result to g/µL, which was subsequently divided by the molar weight of the target sequence and multiplied with 6.022×10^{23} (Avogadro constant). For each sample, we also calculated the quagga mussel ratio as the mean concentration of quagga mussel copies divided by the sum of quagga and zebra mussel copies. All calculations and visualizations of the data were done with the statistical program R (R-Core-Team 2014).

We excluded all replicates of samples and negative controls from the qPCR data set for which the height and shape of melting curves did not match with those of the corresponding standards. Three controls for the DpoCOI3 amplification, could not be excluded by this procedure, but had a higher Cq value than the lowest amplifying standard (Supplementary material Figure S1). These three negative controls and those eDNA samples which showed higher Cq values than the lowest amplifying standards were repeated in standard PCR to confirm false positive and false negative results. None of the negative controls amplified and were thus excluded (Supplementary material Figure S2). For the quantitative analysis of eDNA concentrations we included all samples for which the presence of target DNA fragments was confirmed by standard PCR.

Confirmation of target DNA fragments by sequencing of PCR products:

To confirm the species specificity of the primers we sequenced the PCR products of one lab filtered sample per site and a field filtered sample from Wageningen, Lake IJsselmeer and Lake Markermeer. Singleplex PCRs were run for each primer pair with 2 µL eDNA per reaction. In order to get enough product for sequencing, a second, nested PCR with 30 µL reaction volume was performed (using the same primers as for the Singleplex PCR) for each sample and primer pair with 4 µL PCR product from the first PCR. Each product from the second PCR was checked on an agarose gel, a subsample was purified using the centrifugation protocol of the Wizard SV Gel and PCR Clean-Up System and eluted with 40 µL of nuclease-free water. We sent 15 µL of each purified product to Microsynth (Microsynth, Switzerland) for sequencing. The resulting sequences were aligned in Mega version 6 (Tamura et al. 2013) and compared to COI sequences of zebra mussels and quagga mussels downloaded from Genbank and blasted against the NCBI nucleotide database.

Results

Primer specificity testing:

The DpoCOI3 and DbuCOI3 primer pairs amplified DNA of zebra and quagga mussels in a species specific manner. Neither of the two primer pairs amplified non-target DNA from the mussel tissue samples nor from any of the eDNA samples, which was confirmed by sequencing of PCR products from Singleplex PCRs. Among the 37 zebra mussel COI sequences found on Genbank (Supplementary material Table S3) and which contained both primer binding sites of DpoCOI3, we found one mismatch with the DpoCOI3 forward primer in one single zebra mussel sequence (Accession number: JQ435817, origin: Romania). We also found only one mismatch with the DbuCOI3 reverse primer in one single quagga mussel sequence (Accession number: JQ435816.1, origin: Romania), among the 15 sequences containing both primer binding sites of DbuCOI3. Each primer had three, four or six mismatches with the non-target dreissenid binding site (Table 2). When blasted against the NCBI nucleotide database, both primer pairs showed a similarity of less than 80% with any non-target sequence.

Species detection by standard PCR:

Zebra mussels were detected in eDNA samples from all sites, in almost all pooled field filtered and lab filtered samples (Figure 2). Only one field filtered sample from Lake Markermeer (out of N = 2) and one from Lake IJsselmeer (out of N = 3) did not amplify the zebra mussel target, potentially reflecting very low zebra mussel abundances. The quagga mussel target was detected in all pooled field filtered and lab filtered samples, except the ones collected upstream of Basel. The quagga mussel primers also amplified in the eDNA samples from Basel, where the quagga mussel had not been detected so far. None of our PCR controls, extraction controls or lab filtered or field filtered controls amplified any zebra or quagga mussel DNA, except for three field filtered controls (from Möhlin, Worms and Hanau), for which we found amplification of zebra mussel DNA (data not shown). As we could not exclude the possibility of contamination during filtration in these cases, only lab filtered samples were used for the quantitative PCR, where possible.

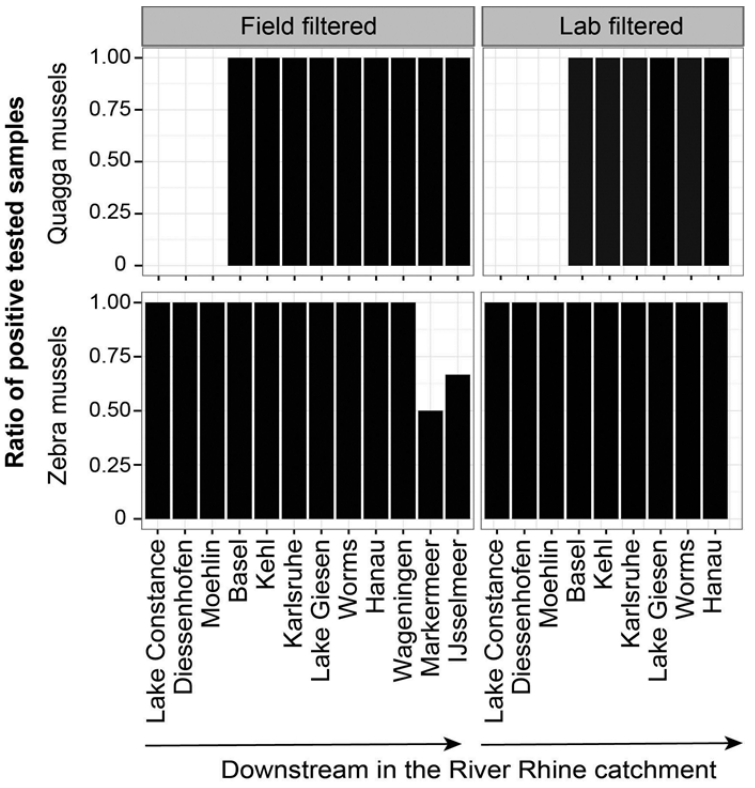

Figure 2. Testing the presence of quagga mussels (upper panel) and zebra mussels (lower panel) on the three eDNA replicates per site (N = 3). Except for Lake Markermeer (N = 2) where one sample had to be discarded. Extracted eDNA samples were analyzed with species specific primers using standard (end-point) PCR. The Y-axis indicates the proportion of positive samples per sampling site. The downstream direction of the River Rhine along which samples haven been collected, is indicated by an arrow.

Quantification of zebra and quagga mussel target DNA fragments by qPCR:

For both targets the lowest standard dilution (6.6×10^{-11} ng/µL for the quagga mussel, 1.5×10^{-10} ng/µL for the zebra mussel) did not amplify in all of the replicates and the limit of quantification (LOQ) was designated as the second lowest standard dilution for both standard curves, which amplified in all replicates (quagga mussel standard: 6.6×10^{-10} ng/µL, Cq value of 31.7 ± SE = 0.37, zebra mussel standard: 1.5×10^{-9} ng/µL, Cq value of 30.8 ± SE = 0.28, see Figure 3). The average LOQ for the quagga mussel target was 8.1 copies per µL (SE = 2.3, equivalent to a mean Cq value of 31.7, SE ± 1.9) while the average LOQ for the zebra mussel target was 12 copies per µL (SE ± 2.6, equivalent to a mean Cq value of 30.7, SE ± 1.2). The quagga mussel standard curve had an amplification efficiency of 98.6% and a slope of −3.39, while the amplification efficiency was 99.0% and the slope −3.37 for the zebra mussel standard curve. We were able to identify by sequencing and blasting all the 24 PCR products which we had amplified with the DpoCOI3 or the DbuCOI3 primer pairs as either zebra mussel or quagga mussel DNA, respectively.

We found a comparable pattern of zebra and quagga mussel presence with qPCR as with PCR presented above. Zebra mussel DNA was detected at all sites, while quagga mussel DNA was present at all sites except those upstream of Basel (Figure 4a). Mean numbers of detected eDNA copies per µL per site and species and corresponding standard errors are shown in Figure 4. In the Rhine in Basel the number of detected eDNA copies per µL was clearly lower for quagga (9.5×10^3 seq/µL, SE ± 1.1×10^3) than for zebra mussels (4.5×10^5 seq/µL, SE ± 8.6×10^4), while in all other locations downstream of Basel the concentration of quagga eDNA copies was higher. The highest concentration of zebra mussel DNA was found in Diessenhofen (2.8×10^6 seq/µL, SE ± 3.9×10^5), where also field samplings of zebra mussels few kilometers upstream showed extremely high densities (Hesselschwerdt et al. 2014). The lowest zebra mussel signals we found in Lake Markermeer and Lake IJsselmeer, agreeing with a survey conducted in 2012 (handpicking on shore, data not shown). As a previous study by Heiler and colleagues (2013) mainly focused on the ratio of quagga mussel abundances to total dreissenid abundances (zebra plus quagga mussels) from field survey data, we also present those ratios calculated

from our qPCR data (Figure 4b). The ratio was still very low for Basel (0.02, SE ± 0.0017) but high for all other sites downstream of Basel, except for Hanau (0.45, SE ± 0.061). Interestingly, for Lake IJsselmeer and in the River Rhine near Worms the ratio was almost 100%, with ratios of 0.991 (SE ± 0.004) and 0.996 (SE ± 0.0036), respectively (Figure 4b).

Discussion

Using an eDNA approach we were able to detect quagga mussels in all sampling sites downstream of Basel and for the first time also in the harbour in Basel (Figure 2). Since 2006 the quagga mussel has rapidly expanded southwards in the Rhine system and has been detected as far south as Karlsruhe in 2007 (Martens et al. 2009). Therefore, the appearance of the quagga mussel in Basel has been expected for several years. However, quagga mussels had not been detected in monitoring programs run by environmental offices and authorities using traditional benthic invertebrate sampling methods including kicknet sampling, surber-sampling and scuba diving in the upper Rhine around Basel (Figure 1).

Our study shows that zebra and quagga mussels can be detected reliably in eDNA samples using standard PCR with the species specific primers used in this study. All of the eDNA samples collected upstream of Basel, where the quagga mussel had not been found by traditional sampling, failed to amplify the quagga mussel target sequence (Figure 2). The only false positives occurred for the zebra mussel, in three field filtered controls, likely due to eDNA cross-contamination during handling of the filters in the field. Nevertheless, cross-contamination between sites is unlikely as we used new or cleaned equipment for each sampling site. The advantage of the field-filtration method is that filters can be frozen directly in the field and water samples do not need to be transported to the lab, during which the eDNA might degrade. Despite the need for careful controls, the detection of mussels with species specific PCR is reliable, inexpensive and less time consuming compared to traditional sampling (Thomsen and Willerslev 2015). Egan et al. (2015) previously demonstrated that eDNA methods reliably detected zebra and quagga mussels in ballast and harbour water samples. However, they used a different set of primers (Mahon et al. 2011) and combined eDNA detection with field-ready light transmission spectroscopy (LTS), a new method which is not used in a standard way yet, neither in research nor in monitoring.

For both species, we estimated a qPCR quantification limit of roughly 10 eDNA copies per µL (Figure 3). This translates to a minimum lower quantification limit of 10×10^6 eDNA copies per L collected water sample, assuming that no eDNA got lost during the sampling, transport, filtration and extraction processes and that there were no PCR inhibiting substances retained in the eDNA extracts. PCR inhibiting substances may be co-extracted with the eDNA and may lead to inaccurate detection or quantification of eDNA (McKee et al. 2015; Sigsgaard et al. 2015). Using the Qiagen PCR Multiplex Master Mix we hoped to mitigate PCR inhibition. Nevertheless, filters clogged easily and retained high amounts of organic and inorganic material in some sites (Worms, Hanau, Wageningen, Lake Markermeer and Lake IJsselmeer) and thus we cannot exclude PCR inhibition completely. In order to reliably detect and control for PCR inhibition, we recommend spiking

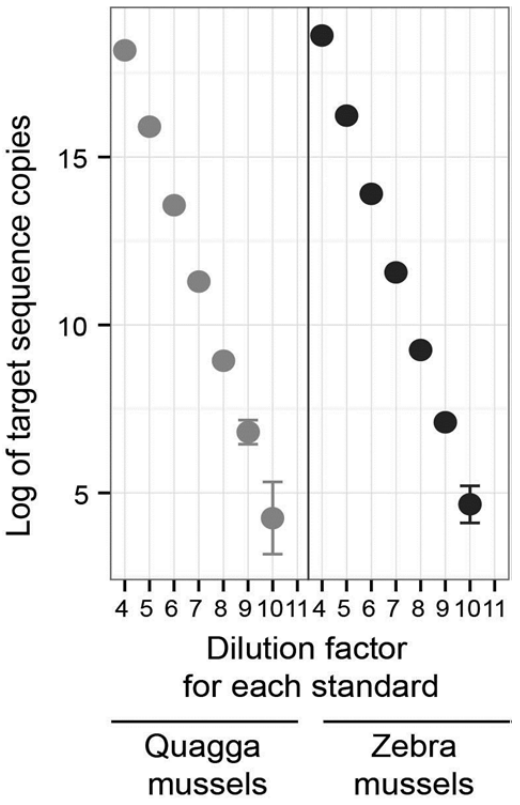

Figure 3. Means of log concentrations (log number of target sequence copies/µL) for each standard dilution of quagga mussel (grey circles) and zebra mussel (black circles) target sequences. The error bars show standard errors of the mean. A dilution factor of 4 represents the highest standard concentration (8×10^9 sequence copies/µL for quagga mussels and 1.1×10^{10} sequence copies/µL for zebra mussels). A dilution factor of 11 represents the lowest standard concentration (0.8 sequence copies/µL for quagga mussels and 1.1 sequence copies/µL for zebra mussels). The lowest concentrated standards of both standard curves with a dilution factor of 11 did not amplify.

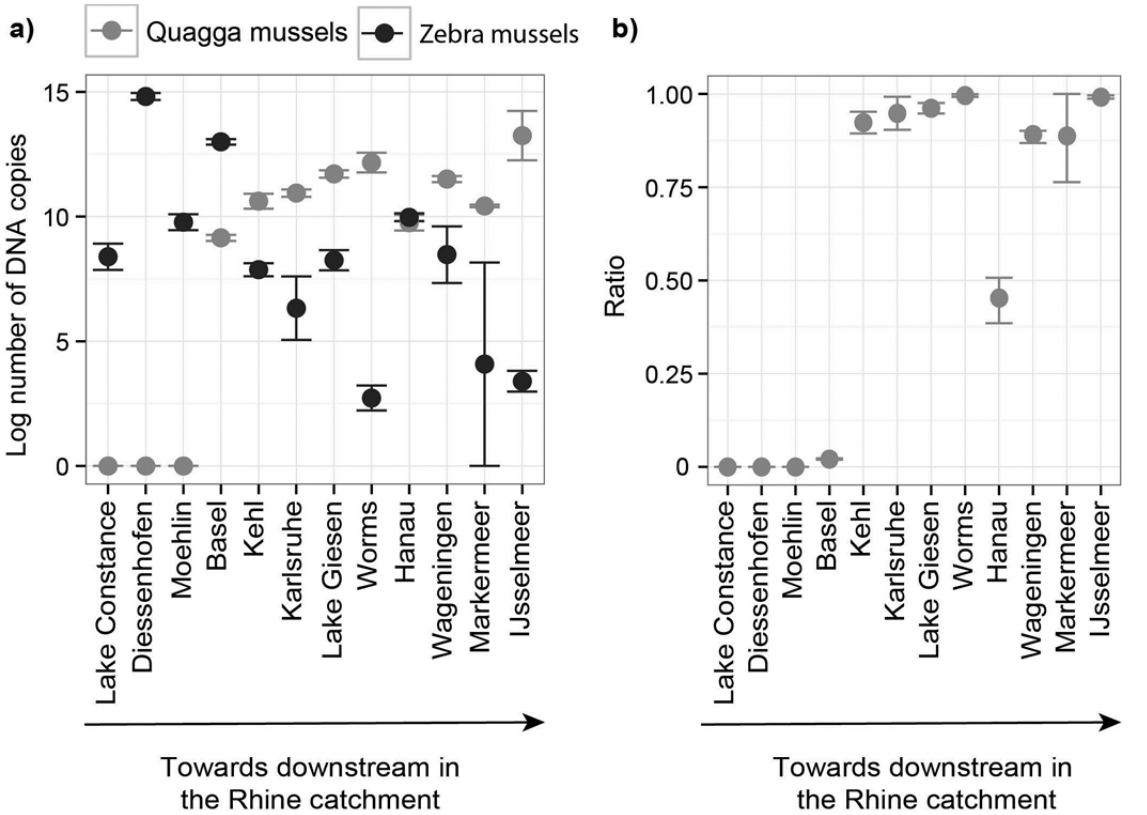

Figure 4. a) $Means_{sample\ replicates}$ of the $mean_{technical\ replicates}$ logarithmic concentrations (log number of target sequence copies/µL) for each sampling site as a proxy for zebra and quagga mussel biomass. The error bars show standard errors of the mean. **b)** Ratio of quagga mussels eDNA concentrations to the total dreissenid eDNA concentration (zebra plus quagga mussels) measured as number of target sequence copies/µL for each sampling site. The error bars show the standard errors of the mean ratio.

of eDNA samples with synthetic DNA oligonucleotides of a known length and concentration to be quantified along with the target eDNA (Goldberg et al. 2016). However, the amplification efficiencies in our qPCR experiment were similarly high for both species and thus the estimated eDNA concentrations may directly be compared between the species and may be used as a proxy for zebra and quagga mussel biomass in the field.

Comparing eDNA concentrations of zebra and quagga mussels with qPCR revealed similar concentration patterns as we had expected based on previous knowledge of zebra and quagga mussel abundances in the field sites. In mesocosm experiments with fish or amphibians, eDNA quantification has been shown to correlate well with known densities or biomass of the target organisms (Takahara et al. 2012; Thomsen et al. 2012). Pilliod et al. (2013) also found strong correlation of eDNA quantification with density estimates from field survey data in two amphibian species. Most field survey data for dreissenids in the River Rhine catchment stem from older sampling campaigns in 2008 and 2009. Knowing that the quagga mussel invasion front has shifted southwards since then, with quagga mussels displacing zebra mussels, this data cannot be directly compared with our eDNA quantifications in a statistical model. Nevertheless, zebra mussel eDNA concentrations were high in the upper Rhine and decreased towards the lower Rhine (Figure 4a), where they were shown to be increasingly displaced by quagga mussels (Heiler et al. 2013). In contrast, quagga mussel eDNA concentrations were lowest in Basel and increased northwards with increasingly long invasion history, except for Hanau and Lake Markermeer (Figure 4a). Also the quagga mussel ratio was still low in Basel where the quagga has invaded most recently, but was close to 100% at sites with longer invasion history. Our zebra and quagga mussel eDNA quantification estimates across the Rhine river catchment strongly suggest that quagga mussels are moving southward and displacing zebra mussels (Figure 4b).

Our qPCR approach revealed relatively small differences between samples from the same site leading to small standard errors and indicating high precision of the method (Figure 4a). The variation was clearly larger for samples with low eDNA concentrations close to the detection limit. Elbrecht and Leese (2015) found a strong correlation between biomass of various invertebrate species and DNA quantification, using a metabarcoding approach. Likely due to primer bias, their amplicon abundance varied strongly between the invertebrate taxa used in their experiment. However, our qPCR standard curve showed similar amplification efficiencies (98.6% and 99% for quagga and zebra mussels respectively) and slopes (−3.39 for quagga compared to −3.37 for zebra mussels, respectively) indicating a low primer bias. Nevertheless, many other factors may confound eDNA quantifications by influencing the formation and decay rates of target eDNA in freshwater systems. For example eDNA shedding depends on the species identity (Mächler et al. 2014), diet of the studied organism and temperature of the environment while decay rates depend on environmental factors such as temperature or light exposure (Klymus et al. 2015). Furthermore, the predominance of different life history stages, reproduction life cycle and seasonality of target organisms may strongly influence the eDNA quantity. For example, we cannot completely exclude that we caught zebra or quagga mussel larvae in our water samples, which may have led to a strong signal in the eDNA quantification. Despite all these confounding factors qPCR may help to investigate the population development over different geographic and temporal scales. We recommend that the use of eDNA quantification as a proxy for zebra and quagga mussel densities needs to be validated in mesocosm experiments with known mussel densities or in comparison with more recent field data specifically assessed for such a study.

Conclusions

Our study shows that eDNA detection with standard PCR is a reliable method for the targeted early detection and surveillance of zebra and quagga mussels. This method is inexpensive, fast if applied for a series of samples from different sampling sites and does not need very complicated equipment, except of a simple PCR-cycler and a gel casting system. In our case, eDNA detection with standard PCR was not only cheaper and simpler but also a more robust method than qPCR. It was less prone to false positives as it has lower sensitivity and also less prone to false negatives as qPCR signals at very low eDNA target concentrations were sometimes ambiguous and had to be confirmed with standard PCR.

We show that eDNA quantification as a proxy for measures of zebra and quagga mussel biomass is a promising technique for the future. Particularly in lotic environments, target eDNA will be washed in and out with certain rates (Jane et al. 2015) and the eDNA signal will possibly only be lost several kilometres downstream of a point source (Deiner and Altermatt 2014). Thus eDNA quantification as a proxy for organism densities may not be appropriate in small scale studies but may apply for the quantification of invasive species on larger geographic scales as presented in our study. Standard PCR and qPCR are powerful tools for the early detection and surveillance of specific species. For routine applications, the two techniques have different advantages and limitations and one of them should be selected depending on the objective of the survey and the methods available. Both techniques are already widely applied to the detection of invasive and parasitic species, for example the crayfish plague (Strand 2013) or parasitic fish diseases such as the proliferative kidney disease or bryozoans as its intermediate hosts (Anderson et al. 1999; Okamura et al. 2011).

In order to manage invasive species, it is important to detect new invaders early on and follow their population development in the early phase of invasion. For the early detection of quagga mussels in Switzerland, we recommend that water samples are taken repeatedly across the year at neuralgic water bodies and assessed with traditional PCR. Sampling sites could be in the upper Rhine or in lakes such as Constance, Geneva or Zürich, which are used intensively for recreational boating and are thus at high risk entry points for the quagga mussel invasion (De Ventura et al. 2016). In the case of invasion, eDNA quantification will then help to follow the population development of zebra and quagga mussels over time and reveal the potential displacement of the zebra mussel populations by the quagga mussel.

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References

Ackerman JD, Sim B, Nichols SJ, Claudi R (1994) Review of the early-life history of zebra mussel (*Dreissena polymorpha*) – Comparisons with marine bivalves. *Canadian Journal of Zoology – Revue Canadienne De Zoologie* 72: 1169–1179, https://doi.org/10.1139/z94-157

Anderson CL, Canning EU, Okamura B (1999) 18S RDNA sequences indicate that PKX organism parasitizes bryozoa. *Bulletin of the European Association of Fish Pathologists* 19(3): 94–97

Arscott DB, Tockner K, Ward JV (2003) Spatio-temporal patterns of benthic invertebrates along the continuum of a braided Alpine river. *Archiv für Hydrobiologie* 158: 431–460, https://doi.org/10.1127/0003-9136/2003/0158-0431

Baldwin BS, Mayer MS, Dayton J, Pau N, Mendilla J, Sullivan M, Moore A, Ma A, Mills EL (2002) Comparative growth and feeding in zebra and quagga mussels (*Dreissena polymorpha* and *Dreissena bugensis*): implications for North American lakes. *Canadian Journal of Fisheries and Aquatic Sciences* 59: 680–694, https://doi.org/10.1139/f02-043

Barbour MT, Gerritsen J, Snyder B, Stribling J (1999) Rapid Bioassessment Protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates and fish. EPA 841-B-99-002 (2nd edition) U.S. Environmental Protection Agency; Office of Water Washington, DC., 1999, 340 pp

Bij de Vaate A, Jazdzewski K, Ketelaars HAM, Gollasch S, Van der Velde G (2002) Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. *Canadian Journal of Fisheries and Aquatic Sciences* 59: 1159–1174, https://doi.org/10.1139/f02-098

Bronnenhuber JE, Wilson CC (2013) Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources* 5: 971–975, https://doi.org/10.1007/s12686-013-9946-0

Deiner K, Altermatt F (2014) Transport Distance of Invertebrate Environmental DNA in a Natural River. *PloS ONE* 9: e88786, https://doi.org/10.1371/journal.pone.0088786

Deiner K, Walser JC, Mächler E, Altermatt F (2015) Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation* 183: 53–63, https://doi.org/10.1016/j.biocon.2014.11.018

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C (2012) Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology* 49: 953–959, https://doi.org/10.1111/j.1365-2664.2012.02171.x

De Ventura L, Weissert N, Tobias R, Kopp K, Jokela J (2016) Overland transport of recreational boats as a spreading vector of zebra mussel *Dreissena polymorpha*. *Biological Invasions* 18: 1451–1466, https://doi.org/10.1007/s10530-016-1094-5

Diggins TP (2000) Comparative filtration rate and sensitivity to disturbance of *Dreissena bugensis* and *D. polymorpha*. IAGLR Conference Program and Abstracts 43: A–35

Dougherty MM, Larson ER, Renshaw MA, Gantz CA, Egan SP, Erickson DM, Lodge DM (2016) Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology* 53: 722–732, https://doi.org/10.1111/1365-2664.12621

Egan SP, Grey E, Olds B, Feder JL, Ruggiero ST, Tanner CE, Lodge DM (2015) Rapid molecular detection of invasive species in ballast and harbor water by integrating environmental DNA and light transmission spectroscopy. *Environmental Science and Technology* 49: 4113–4121, https://doi.org/10.1021/es5058659

Elbrecht V, Leese F (2015) Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass—Sequence Relationships with an Innovative Metabarcoding Protocol. *PloS ONE* 10: e0130324, https://doi.org/10.1371/journal.pone.0130324

Fulton T (2012) Setting Up an Ancient DNA Laboratory. In: Shapiro B, Hofreiter M (eds), Ancient DNA. Methods in Molecular Biology. Humana Press, pp 1–11, https://doi.org/10.1007/978-1-61779-516-9_1

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science* 32: 792–800, https://doi.org/10.1899/13-046.1

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE, Taberlet P (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution* 7: 1299–1307, https://doi.org/10.1111/2041-210X.12595

Heiler KCM, Bij de Vaate A, Ekschmitt K, Oheimb PV von, Albrecht C, Wilke T (2013) Reconstruction of the early invasion history of the quagga mussel (*Dreissena rostriformis bugensis*) in Western Europe. *Aquatic Invasions* 8: 53–57, https://doi.org/10.3391/ai.2013.8.1.06

Hesselschwerdt J, Muerle U, Werner S, Rey P (2014) Gewässer-monitoring im Hochrhein [Data from the monitoring on aquatic biota in the upper River Rhine]. Hydra-Institute, Konstanz

Higgins SN, Vander Zanden MJ (2010) What a difference a species makes: a meta-analysis of dreissenid mussel impacts on freshwater ecosystems. *Ecological Monographs* 80: 179–196, https://doi.org/10.1890/09-1249.1

Imo M, Seitz A, Johannesen J (2010) Distribution and invasion genetics of the quagga mussel (*Dreissena rostriformis bugensis*) in German rivers. *Aquatic Ecology* 44: 731–740, https://doi.org/10.1007/s10452-009-9311-2

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, flow and PCR inhibition: EDNA dynamics in two headwater streams. *Molecular Ecology Resources* 15: 216–227, https://doi.org/10.1111/1755-0998.12285

Jerde CL, Chadderton WL, Mahon AR, Renshaw MA, Corush J, Budny ML, Mysorekar S, Lodge DM (2013) Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences* 70: 522–526, https://doi.org/10.1139/cjfas-2012-0478

Kinzelbach R (1992) The main features of the phylogeny and dispersal of the zebra mussel (*Dreissena polymorpha*). In: Neumann D, Jenner HA (eds), Zebra Mussel *Dreissena Polymorpha* – Ecology, Biological Monitoring and First Applications in the Water Quality Management. Limnologie Aktuell. Gustav Fischer Verlag, Stuttgart, pp 5–17

Klymus KE, Richter CA, Chapman DC, Paukert C (2015) Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation* 183: 77–84, https://doi.org/10.1016/j.biocon.2014.11.020

Leuven RSEW, Collas FPL, Koopman KR, Matthews J, Van der Velde G (2014) Mass mortality of invasive zebra and quagga mussels by desiccation during severe winter conditions. *Aquatic Invasions* 9: 243–252, https://doi.org/10.3391/ai.2014.9.3.02

Lockwood JL, Hoopes MF, Marchetti MP (2007) Invasion ecology. Blackwell Pub, Malden, MA, 304 pp

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

Mahon AR, Barnes MA, Senapati S, Feder JL, Darling JA, Chang HC, Lodge DM (2011) Molecular Detection of Invasive Species in Heterogeneous Mixtures Using a Microfluidic Carbon Nanotube Platform. *PloS ONE* 6: e17280, https://doi.org/10.1371/journal.pone.0017280

Martens A, Grabow K, Schoolmann G (2009) Die Quagga-Muschel *Dreissena rostriformis bugensis* am Oberrhein: der Beginn einer Invasion [The Quagga mussel *Dreissena rostriformis bugensis*

in the upper Rhine, the beginning of an invasion]. Annual assemply of the German Limnological Society (DGL) 2008, Hardegsen, Lauterbornia, Hardegsen, p 4

Matthews J, Schipper AM, Hendriks AJ, Yen Le TT, Bij de Vaate A, Van der Velde G, Leuven RSEW (2015) A dominance shift from the zebra mussel to the invasive quagga mussel may alter the trophic transfer of metals. *Environmental Pollution* 203: 183–190, https://doi.org/10.1016/j.envpol.2015.03.032

Matthews J, Van der Velde G, Bij de Vaate A, Collas FPL, Koopman KR, Leuven RSEW (2014) Rapid range expansion of the invasive quagga mussel in relation to zebra mussel presence in The Netherlands and Western Europe. *Biological Invasions* 16: 23–42, https://doi.org/10.1007/s10530-013-0498-8

McKee AM, Spear SF, Pierson TW (2015) 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. *Biological Conservation* 183: 70–76, https://doi.org/10.1016/j.biocon.2014.11.031

Mills EL, Rosenberg G, Spidle AP, Ludyanskiy M, Pligin Y, May B (1996) A review of the biology and ecology of the quagga mussel (*Dreissena bugensis*), a second species of freshwater dreissenid introduced to North America. *American Zoologist* 36: 271–286, https://doi.org/10.1093/icb/36.3.271

Okamura B, Hartikainen H, Schmidt-Posthaus H, Wahli T (2011) Life cycle complexity, environmental change and the emerging status of salmonid proliferative kidney disease. *Freshwater Biology* 56: 735–753, https://doi.org/10.1111/j.1365-2427.2010.02465.x

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences* 70: 1123–1130, https://doi.org/10.1139/cjfas-2013-0047

Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52: 273–288, https://doi.org/10.1016/j.ecolecon.2004.10.002

R-Core-Team (2014) Development Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0

Roe SL, MacIsaac HJ (1997) Deepwater population structure and reproductive state of quagga mussels (*Dreissena bugensis*) in Lake Erie. *Canadian Journal of Fisheries and Aquatic Sciences* 54: 2428–2433, https://doi.org/10.1139/f97-151

Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, Leemans R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Sykes MT, Walker BH, Walker M, Wall DH (2000) Global Biodiversity Scenarios for the Year 2100. *Science* 287: 1770–1774, https://doi.org/10.1126/science.287.5459.1770

Sigsgaard EE, Carl H, Møller PR, Thomsen PF (2015) Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation* 183: 46–52, https://doi.org/10.1016/j.biocon.2014.11.023

Strand DA (2013) Environmental DNA monitoring of the alien crayfish plague pathogen *Aphanomyces astaci* in freshwater systems. University of Oslo, department of biosciences: 188

Strayer DL (2009) Twenty years of zebra mussels: lessons from the mollusk that made headlines. *Frontiers in Ecology and the Environment* 7: 135–141, https://doi.org/10.1890/080020

Stucki P (2010) Methoden zur Untersuchung und Beurteilung der Fliessgewässer: Makrozoobenthos-Stufe F [Methods for the assessment and evaluation of streams: Macrozoobenthos - level F]. Umwelt-Vollzug 1026, Bern, Switzerland, 2010, 61 pp

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. *PloS ONE* 7: e35868, https://doi.org/10.1371/journal.pone.0035868

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30(12): 2725–2729, https://doi.org/10.1093/molbev/mst197

Therriault TW, Orlova MI, Docker MF, Macisaac HJ, Heath DD (2005) Invasion genetics of a freshwater mussel (*Dreissena rostriformis bugensis*) in eastern Europe: high gene flow and multiple introductions. *Heredity* 95: 16–23, https://doi.org/10.1038/sj.hdy.6800691

Thomsen PF, Kielgast JOS, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP, Orlando L, Willerslev E (2012) Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* 21: 2565–2573, https://doi.org/10.1111/j.1365-294X.2011.05418.x

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

Vanderploeg HA, Nalepa TF, Jude DJ, Mills EL, Holeck KT, Liebig JR, Grigorovich IA, Ojaveer H (2002) Dispersal and emerging ecological impacts of Ponto-Caspian species in the Laurentian Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences* 59: 1209–1228, https://doi.org/10.1139/f02-087

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *PloS ONE* 8: e59520, https://doi.org/10.1371/journal.pone.0059520

Zhulidov AV, Kozhara AV, Scherbina GH, Nalepa TF, Protasov A, Afanasiev SA, Pryanichnikova EG, Zhulidov DA, Gurtovaya TY, Pavlov DF (2010) Invasion history, distribution, and relative abundances of *Dreissena bugensis* in the old world: a synthesis of data. *Biological Invasions* 12: 1923–1940, https://doi.org/10.1007/s10530-009-9641-y

Supplementary material

The following supplementary material is available for this article:

Figure S1. Cq values for all pooled samples, controls, extraction controls and standards.

Figure S2. Verification of ambiguous qPCR-amplifications by standard PCR.

Table S1. eDNA sampling sites with geographic information, sampling date, filtration method, expected quagga mussel presence and sample ID.

Table S2. Geographic information of eDNA sampling sites.

Table S3. List of quagga and zebra mussel sequences, which we downloaded from Genbank.

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