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Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species

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Abstract: Accurate knowledge of the distribution of rare, indicator, or invasive species is required for conservation and management decisions. However, species monitoring done with conventional methods may have limitations, such as being laborious in terms of cost and time, and often requires invasive sampling of specimens. Environmental DNA (eDNA) has been identified as a molecular tool that could overcome these limitations, particularly in aquatic systems. Detection of rare and invasive amphibians and fish in lake and river systems has been effective, but few studies have targeted macroinvertebrates in aquatic systems. We expanded eDNA techniques to a broad taxonomic array of macroinvertebrate species in river and lake systems. We were able to detect 5 of 6 species (*Ancylus fluviatilis*, *Asellus aquaticus*, *Baetis buceratus*, *Crangonyx pseudogracilis*, and *Gammarus pulex*) with an eDNA method in parallel to the conventional kicknet-sampling method commonly applied in aquatic habitats. Our eDNA method showed medium to very high consistency with the data from kicknet-sampling and was able to detect both indicator and nonnative macroinvertebrates. Furthermore, our primers detected target DNA in concentrations down to 10^{-5} ng/μL of total extracted tissue DNA in the absence of background eDNA in the reaction. We demonstrate that an eDNA surveillance method based on standard PCR can deliver biomonitoring data across a wide taxonomic range of macroinvertebrate species (Gastropoda, Isopoda, Ephemeroptera, and Amphipoda) in riverine habitats and may offer the possibility to deliver data on a more refined time scale than conventional methods when focusing on single or few target species. Such information based on nondestructive sampling may allow rapid management decisions and actions.

Key words: eDNA, kicknet sampling, lotic systems, cytochrome oxidase I, water quality assessment, EPT, Amphipoda

Knowledge about a species' distribution is important in ecology, conservation biology, and invasion biology. Freshwater systems are of particular interest because they are among the most biodiverse habitats on earth (Vörösmarty et al. 2010), and the high diversity is important to maintain ecosystem services. However, ¼ of known worldwide freshwater species are listed as threatened or regionally extinct (IUCN 2012), and distribution and abundance of many species is rapidly changing. Thus, knowledge of the distribution of both native and nonnative species is urgently needed to understand and protect freshwater systems worldwide. Aquatic species, and especially aquatic invertebrates, are notoriously difficult to inventory because of their small size and often low population densities, their patchy distribution, or the complexity in their use of the habitat at different life stages (Barbour et al. 1999).

Many traditional monitoring methods in aquatic systems, such as kicknet-sampling, are laborious in terms of cost and time (Barbour et al. 1999, Stucki 2010), especially for macroinvertebrates, which are small and can occur at very different abundances. Monitoring of a few targeted species also often involves extensive sorting and processing steps depending on specific taxonomic expertise. Furthermore, classic sampling methods depend on the collection of specimens, are hard to standardize, and are often limited in taxonomic resolution or by the impossibility of identifying immature or damaged specimens (Pfrender et al. 2010, Baird and Hajibabaei 2012, Deiner et al. 2013). As a consequence, in many bioassessments, taxa are identified only at a genus or family level, whereas for conservation or management decisions, information on the level of species would be valuable, especially for nonnative or endangered species. Several

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investigators have shown that DNA sequence-based approaches in combination with next generation sequencing can be used to assess biodiversity from arthropod bulk samples (Hajibabaei et al. 2011, Yu et al. 2012). However, in all of these approaches one must sample the animals, which is a time-consuming step. Novel methods that can overcome these limitations are needed, especially for monitoring rare, indicator, or invasive species. Use of such methods would require high sensitivity for targeted individual species and standardized protocols. Furthermore, the method should be able to detect species that are rare or occur at low densities, such as species at invasion fronts or endangered species (MacKenzie et al. 2005, Baird and Hajibabaei 2012).

Environmental DNA (eDNA) is a molecular approach that can overcome some of the above-mentioned limitations because it allows detection of organisms based only on collection of their DNA from the medium in which they live (Baird and Hajibabaei 2012; Yoccoz 2012). We advocate the use of standard polymerase chain reaction (PCR) with specific primers as a better approach to study the occurrence of one or a few target species than more expensive next generation sequencing techniques, which may be better for whole-community assessments. In eDNA surveillance, species are detected using DNA that they shed into the environment in many ways, such as feces, hair, or epidermal cells (Lydolph et al. 2005). The application of eDNA to detection of aquatic macroinvertebrate species may allow monitoring of these species at a high temporal resolution, could be standardized through adoption of strict molecular protocols, and would not depend on specific taxonomic expertise or the collection of specimens once the assays for the species' eDNA were developed and validated. eDNA already has been used to detect various species in aquatic systems, such as the invasive American bullfrog (*Lithobates catesbeianus*; Ficetola et al. 2008, Dejean et al. 2011), invasive Asian carps (*Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis*; Jerde et al. 2011), and rare frog and salamander species (Goldberg et al. 2011).

In many aquatic systems, macroinvertebrates make up a very large proportion of the local diversity. Their inclusion in biomonitoring is essential, and they are well studied and commonly used in biodiversity studies in aquatic systems (Heino et al. 2005, Altermatt 2013). However, until now, macroinvertebrates have been monitored mainly by sampling specimens with kicknet, Surber sampler, or similar methods (Barbour et al. 1999, Stucki 2010, Altermatt et al. 2013), and only a few investigators have used an eDNA approach. For example, Thomsen et al. (2011) detected 2 species (the dragonfly *Leucorrhinia pectoralis* and the tadpole shrimp *Lepidurus apus*) in ponds, and Goldberg et al. (2013) detected the mudsnail *Potamopyrgus antipodarum* in river systems. A general demonstration of the use and suitability of the eDNA method across further invertebrate taxa is lacking. Such a demonstration would be especially desirable for Ephemeroptera, Plecoptera, Trichoptera (EPT) taxa or for amphipods, which are commonly used for freshwater quality assessments or in ecotoxicology, respectively (Barbour et al. 1999, Heino et al. 2005, Stucki 2010).

We designed primer pairs for our study region to target 6 macroinvertebrate species representing ecological indicator species (*Gammarus pulex*, *Asellus aquaticus*, *Ancylus fluviatilis*, and *Tinodes waeneri*), nonnative species (*Crangonyx pseudogracilis*), and a Red-list species classified as vulnerable (*Baetis buceratus*; Lubini et al. 2012) (Table 1). We compared the performance of the eDNA and conventional kicknet-sampling methods for detecting these species in 14 sites in rivers and lakes (Fig. 1). We conducted experiments with a subset of these species to test the specificity and sensitivity of our primers. Specifically, we estimated the potential detection limit of our species in water by diluting known amounts of tissue-derived DNA extracts in DNA-free water. Last, we tested the sensitivity reduction of 2 of these primer pairs in presence of nontargeted DNA.

METHODS

Study sites, sampling, and targeted species

The study was carried out in river and lake habitats in the canton of Zurich in the northeastern part of Switzerland (Fig. 1, Table S1). All study sites are natural water bodies and belong to 2 independent drainages (Glatt River and Limmat River, which drain Lake Greifensee and Lake Zurich, respectively). For the eDNA surveillance method, we collected 2 water samples/site in 1-L sterile octagonal polyethylene terephthalate bottles (VWR International, Radnor, Pennsylvania) by dipping the mouth of the bottle just below the surface of the water near the edge of the water body. We placed water bottles in an ice-filled container during transport and stored them at −20°C until processed. Maximum transport time was 5 h. Immediately after taking water samples, we collected macroinvertebrates with standard kicknet sampling following federal and cantonal guidelines (Stucki 2010, Altermatt 2013; Appendix S1). Thereby, we could compare detection of macroinvertebrates using the novel eDNA surveillance method with the traditional kicknet sampling (for a description of the workflows, see Fig. 2). We took 8 independent kicknet samples per site to reflect the different microhabitats at each study site and pooled them to get an accurate and robust presence/absence measure for all macroinvertebrates (Stucki 2010, Appendix S1).

We choose a range of 6 species belonging to 3 major classes of invertebrates (Table 1) for which we compared the eDNA surveillance method with the conventional kicknet method. We chose species that live in rivers and lakes and are either rare or belong to indicator groups used for water-quality assessment in the study area (von der Ohe et al. 2007). We chose species that had been detected in the study area by the conventional biodiversity monitoring conducted by

Table 1. Overview of the study species.

Class	Order	Family	Species	Habitat type[a]	Indicator group[b]	Taxonomic key used
Crustacea	Amphipoda	Crangonyctidae	*Crangonyx pseudogracilis*[c]	Lentic, lotic	No	Eggers and Martens 2001
Crustacea	Amphipoda	Gammaridae	*Gammarus pulex*	Lotic	Yes	Eggers and Martens 2001
Crustacea	Isopoda	Asellidae	*Asellus aquaticus*	Lentic, lotic	Yes	Tachet et al. 2000
Gastropoda	Basommatophora	Ancylidae	*Ancylus fluviatilis*	Lentic, lotic	Yes	Glöer and Meier-Brook 1998
Insecta	Ephemeroptera	Baetidae	*Baetis buceratus*	Lotic	Yes	Studemann et al. 1992
Insecta	Trichoptera	Psychomyiidae	*Tinodes waeneri*	Lentic, lotic	Yes	Waringer and Graf 1997

[a] Data on habitats are based on monitoring results of the Office of Waste, Water, Energy and Air (WWEA) from 1995–2012
[b] Indicator groups are based on Stucki (2010)
[c] Denotes a nonnative species

the Office of Waste, Water, Energy, and Air (WWEA/AWEL) of the Canton of Zurich over the years 1995–2011 (AWEL 2012) and were known to be part of the regional species pool.

Primer development and tests of sensitivity and specificity

We chose the cytochrome *c* oxidase subunit I (COI) gene to develop primer pairs for our study species (Thomsen et al. 2011, Mahon et al. 2013). We targeted COI because mitochondrial genes have many more copies per cell than nuclear genes (Mills et al. 2000) and, thus, are more likely to be detected. Furthermore, the COI sequence is generally used and advocated for identification of macroinvertebrate species from aquatic systems (Deiner et al. 2013), and existing sequence data can be used for primer development. We gathered sequence data from GenBank for each of the

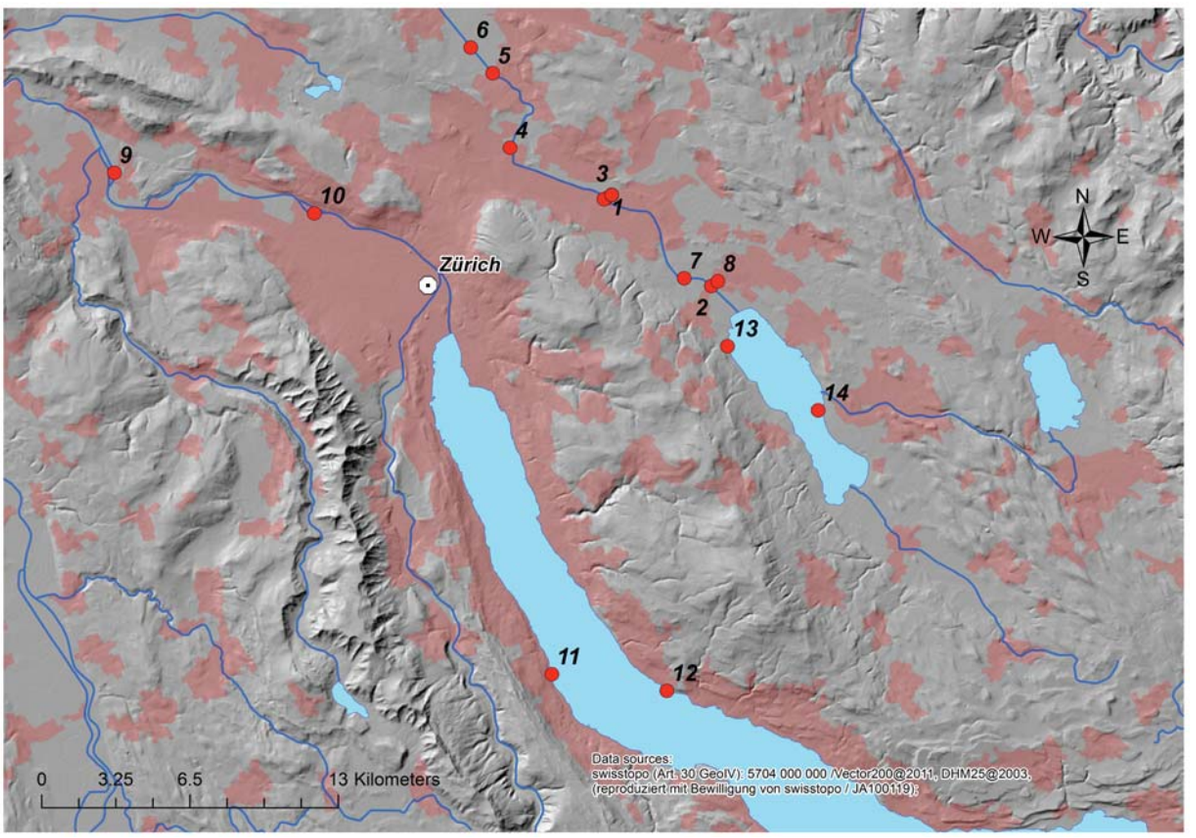

Figure 1. Locations of sampling sites in the northeastern part of Switzerland. All sites were in the drainage systems of the river Glatt and river Limmat and Lake Greifensee and Lake Zurich, respectively. Blue areas represent main water bodies and rivers, whereas red areas represent settlements in the relief map (gray). Numbers correspond to sites listed in Table S1.

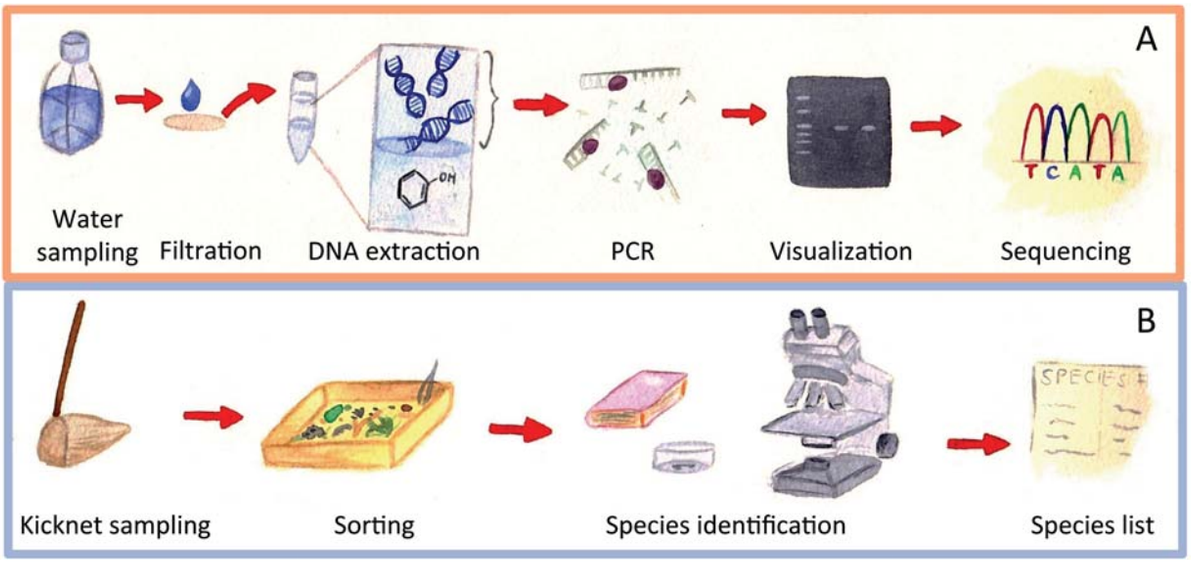

Figure 2. Illustrative overview of the workflow of the 2 surveillance methods. A.—Environmental DNA (eDNA) method (from left to right): collection of water, water filtration, DNA extraction, polymerase chain reaction (PCR), visualization of products on a gel, and sequencing, eventually leading to a list of positive or negative detections of target species. B.—Conventional kicknet method: kicknet sampling, sorting, and morphological identification of species, eventually leading to a species list present at a sampling site.

target species (Benson et al. 2012; Table S2). For each target species, we aligned COI sequences using Sequencher® (version 4.9; Gene Codes, Ann Arbor, Michigan) to observe intraspecific conserved regions. We also aligned sequences of related species to compare intra- and interspecific differences. We selected sequence regions with 180 to 450 base pairs (bp) that showed low intraspecific divergence and high interspecific divergence. We developed primer pairs with amplicon sizes of ~200 bp, which is a size that has been detected successfully for other species (Jerde et al. 2011, Mahon et al. 2013). We acknowledge that eDNA in water is probably a mixture of cellular and extracellular DNA (Taberlet et al. 2012) and can vary in fragment length once concentrated and extracted. Detection with fragments that are too short (<100 bp for COI) may hinder adequate species identification and probably depends on the genetic region used for identification (Meusnier et al. 2008). Therefore, detection of species based on longer fragments of DNA from a water sample allows more accurate species identification and possibly detection of DNA recently released to the environment because the DNA has not had time to degrade into small fragments (Dejean et al. 2011, Thomsen et al. 2011, Goldberg et al. 2013). Primers were designed with Primer3 (version 0.4.0; Rozen and Skaletsky 1999) software using the selected 180- to 450-bp region and the default parameters of the program. When the selected region did not return primers with default parameters, we designed primers by eye from the alignments (Table S3). We tested the specificity of all designed primers with the software Primer-BLAST with default settings (Ye et al. 2012; see Table S4 for results).

To confirm primer amplification success in the target species, we ran standard PCRs with our designed primers on DNA extracts from 2 individuals of each species and sequenced the amplified products. DNA was extracted from tissue of each species using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's spin-column protocol. All PCR mixtures contained 1 × buffer (Roche, Basel Switzerland), 0.18 mM dNTP, 1 × BSA, 0.05 U/μL Taq (Roche, Switzerland), 2.0 mM $MgCl_2$, 0.5 μM of each primer (Table S3), and 1 μL of DNA (1.04–108 ng/μL) in a total reaction volume of 15 μL. Quantification of DNA concentrations was confirmed with a Qubit® (1.0; Life Technologies, Carlsbad, California) fluorometer following recommended protocols for the broad-range DNA concentration kit (Life Technologies). The manufacturer reported ≤3% variation of repeated sample testing. The PCR thermal-cycling program started with a denaturation step at 95°C for 4 min and was followed by 35 cycles of 95°C for 30 s, 30 s at the individual annealing temperature for each primer pair (Table S3), and 1 min at 72°C. A final extension of 5 min at 72°C was done before the PCR was paused at 10°C until removed. We visualized PCR products using gel electrophoresis and photo-documented gels with ultraviolet light on a 1.4% agarose gel either stained with ethidium bromide or GelRed™ (Biotium, Hayward, California). Only single-banded, positive amplicons that we could directly sequence were used for further analyses. We cleaned each positive PCR product with Exo I Nuclease (EXO I) and Shrimp Alkaline Phosphatase (SAP) (Thermo Fisher Scientific, Waltham, Maryland) to remove leftover primers and dNTPs from the previous PCR. The master mix consisted of 1.6 U/μL

Exo I and 0.15 U/μL SAP in a total volume of 1 μL, which was then added to 7.5 μL of the PCR product. Products were heated to 37°C for 15 min and followed by 15 min at 80°C. We did sequencing in both directions with the BigDye® Terminator (version 3.1) system on 3730 × l DNA Analyzer following manufacturers' protocols (Applied Biosystems, Foster City, California). We aligned and edited sequences for each PCR product with Sequencher. To confirm the species identity of the band amplified from extracted DNA from tissues, we aligned sequences to those used for primer design and compared sequences to the nucleotide database on GenBank using default settings in Basic Local Alignment Search Tool (BLAST; Benson et al. 2012). We could not test primer pairs against all closely related species, but we checked whether sequence databases covered COI sequences for all closely related species (i.e., species within same the genus or family) occurring in the regional species pool (Table S5) and, thus, would align when we entered our sequences. This approach (i.e., sequencing all bands) is more time-intensive than inferring species presence from positive PCR amplicons only, but avoids false-positive detection of other (possibly related) species, which may yield positive PCR products of similar length, but with a different COI sequence.

To test the sensitivity of our primers, we tested a dilution series of DNA extracts from 2 species (*B. buceratus*, *C. pseudogracilis*). We diluted extracted DNA to confirm primer amplification success for these 2 species to 1 ng/μL. We then did a serial dilution to create a DNA concentration range of 0.1 ng/μL to 10^{-8} ng/μL. We used each dilution as template in a PCR for final concentrations ranging from 1.08×10^{-1} to 1.04×10^{-8} ng/μL. We ran 3 PCR replicates/concentration. PCR and sequencing to confirm products were carried out as described above except that 50 cycles instead of 35 were used for PCR amplification because we expected low DNA concentrations. We analyzed detection rate of the individual species with a generalized linear model (GLM), with dilution rate and species identity as predictor variables. The binary response variable described detection success or failure. We used a quasibinomial link function because of over-dispersion in the model (Crawley 2012). We started with a full model including interactions and subsequently simplified the model. We selected the best model with an *F*-significance test. All statistical analyses were conducted with the program R (version 2.15.3; R Project for Statistical Computing, Vienna, Austria).

Detection of species from eDNA and conventional methods

We filtered 280 to 300 mL of water from each site onto a single 0.7-μm glass fiber filter 4 times (Whatman International Ltd., Maidstone, UK). We processed 4 filters for each sampling site independently. The filter was housed in a filter case (Swinnex, EMD Millipore Co., Billerica, Massachusetts) to which we could attach a disposable 20-mL syringe. We drew water into the syringe and pushed it through the filter in the housing. We repeated this step until the desired filtered volume was reached. Filtration setup was followed as described by Deiner and Altermatt (2014). After filtration, we removed the filter from the filter housing, rolled it with tweezers, and placed it in a 1.5-mL tube. We stored all filters at –20°C until extraction. We treated all equipment used for filtration with 10% household bleach and sterilized with an ultraviolet light treatment for 30 min after handling samples from a site. We extracted DNA from each filter with a modified cell-lysis, phenol chloroform isoamyl procedure followed by ethanol precipitation of environmental DNA captured on filter as described by Deiner and Altermatt (2014).

We tested for the presence of DNA from target species with our designed primers by PCR amplification, product visualization, and sequencing of amplified products with the same protocol as described for primer tests on tissue-extracted DNA, except that we used 50 PCR cycles instead of 35, and we added 2 μL of eDNA template to each reaction. In a few cases, we used the sequence from only the forward or reverse to confirm presence (indicated with an asterisk in Table S6). If a sequenced amplicon could not be confirmed in both directions, but 1 direction met our strict protocol for confirmation, we counted the case as a positive detection and reported the shorter fragment from only 1 direction. We first tested for presence of every species with eDNA derived from extraction of 280 mL water and triplicate PCR. When detection for some species (*A. fluviatilis*, *C. pseudogracilis*, and *T. waeneri*) was not optimal, we subsequently optimized PCR protocols by performing a temperature and $MgCl_2$-gradient on tissue extracted and on environmental DNA to improve specificity. Second, we repeated PCR on eDNA extracted from a larger volume of water (900 mL; 3 extractions of 300 mL water each were pooled; for details see Table S1) and replicated PCR 5 times for each species at each site. We analyzed this larger total volume (900 mL vs the initial 280 mL) in a 2nd step after realizing that this 4 × increase in volume would allow us to minimize the risk of false-absence detections (KD, J. C. Walser, EM, and FA, unpublished data). For all subsequent analyses, we pooled positive detections from the 2 volumes of water. Therefore, the presence/absence of species identified from eDNA is based on a total of 1180 mL of water extracted/site (280 + 900 mL).

We defined a species as present at a site when ≥1 PCR replicate from either volume of water showed positive amplification that could be confirmed by sequencing the amplified product. PCR is a highly stochastic process, and PCR replication in present eDNA studies ranges from 3 to 8 (Deiner and Altermatt 2014). For sequencing confirmation of a detected species, we required that the following criteria be met: 1) the generated sequences must align to the GenBank sequences used in primer design, and 2) the generated sequence must match the expected species with a high score (>99% maximum identity and >100% query coverage, except

for *B. buceratus*) when compared against the National Center for Biotechnology Information (NCBI) nucleotide database using default settings in BLAST (Benson et al. 2012). For *B. buceratus*, only 1 sequence was available on GenBank. This sequence was delivered from a specimen presumably collected in the Czech Republic. Extracted DNA from a specimen collected in our study region matched this sequence with 100% query coverage but with 98% maximum identity. Use of a 2%-dissimilarity threshold allows for intraspecific diversity. This level of dissimilarity can occur in species with geographical isolation (Hebert et al. 2003). However, all water-derived DNA sequences for *B. buceratus* matched our tissue-derived DNA sequence with ≥99.5% maximum identity.

To identify invertebrates sampled with the kicknet method, we first presorted macroinvertebrates into higher taxonomic groups and then identified them to species level based on morphology with the aid of dichotomous taxonomic keys (Studemann et al. 1992, Waringer and Graf 1997, Glöer and Meier-Brook 1998, Tachet et al. 2000, Eggers and Martens 2001; Table 1). We compared detection rates of the kicknet sample and eDNA surveillance methods based on presence with the kicknet method, presence with the eDNA method, and their overlap. All eDNA presence data used in the comparison (Table S7) were confirmed by sequencing the PCR product (Table S6). The total number of sites used was in accordance to the expected habitat type (n = 10 for species living in lotic habitat only and n = 14 for species living in lentic and lotic habitats).

Laboratory precautions

eDNA is expected to occur at low density. Therefore, we took precautions similar to those used in ancient DNA protocols in all of our laboratory routines to avoid contamination (Fulton 2012). Filtrations, extractions, and prePCR work were done in a DNA-clean laboratory where no post-PCR products and extracted target-species DNA from tissues entered the room. Researchers were required to wear full-body protective gear. Negative filter, extraction, and PCR controls always were run in parallel with the samples and showed no amplifications. Equipment, such as the laminar-flow hood and pipettes were cleaned regularly with 10% household bleach and sterilized with a 30-min ultraviolet-light treatment. In addition, we applied a multitube approach and replicated PCR reactions ≥3 times (and 8 times for all eDNA detections), each with its own negative control (Taberlet et al. 1996).

RESULTS

Primer development and tests of sensitivity and specificity

We were able to amplify the COI region of all 6 species from tissue-derived DNA with our set of primer pairs designed from pre-existing data from GenBank. Furthermore, we confirmed primer amplification success at low target DNA concentrations via specificity tests for 2 species (Fig. 3). Detection rate was significantly affected by the dilution of DNA in DNA-free water and species identity (Table 2, Fig. 3). Detection success was 100% for the 2 tested primer pairs at target DNA concentrations >~10^{-5} ng/µL and dropped to 0% detection success at lower DNA concentrations (based on the GLM prediction) for all primers. The GLM model predicted a sudden decrease of detectability at ~10^{-6} to 10^{-8} ng/µL target DNA (Table 2, Fig. 3).

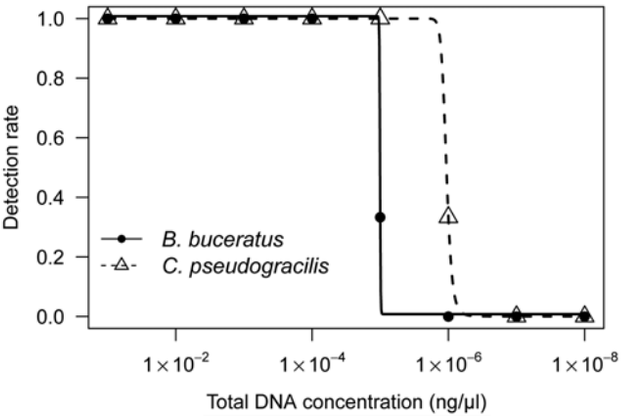

Figure 3. Correlation of detection rate and concentration of total DNA before polymerase chain reaction (PCR) for *Baetis buceratus* and *Crangonyx pseudogracilis*. Detection rate is given as proportion of positive bands across 3 PCR replicates. Lines are predictions of a generalized linear model. The detection rates for DNA concentrations from 10^{-1} to 10^{-5} ng/µl are all 1.0, but lines were slightly vertically displaced for better visibility.

Our primer pairs were not completely species-specific (Table S4) and amplified some nontarget species. However, most of these nontarget species had geographic distributions that did not overlap our study area. To avoid the occurrence of false-positive detections completely, we subsequently sequenced every positive PCR amplification. We used only records based on a sequencing confirmation. There-

Table 2. Generalized linear model (GLM) on the effect of known target DNA concentration and species identity on polymerase chain reaction (PCR) detection success of species-specific environmental DNA (eDNA) in water. Proportion of detection was used as response variable (odds ratio). GLM was done with quasibinomial error distribution and subsequent *F*-significance testing. df = degrees of freedom.

Effect	df	Deviance	Residual df	Residual deviance	*F*	*p*
Dilution	1	49.5	14	7.3	946828	<0.001
Species identity	1	7.2	13	0.001	139195	<0.001
Null			15	56.8		

by, we are certain that positive PCR amplicons used for our analysis reflect detection of eDNA of our target species.

Method comparison

The 6 study species were found with the conventional kicknet method in 3 to 9 of the 14 sampling localities (Fig. 4). We were able to detect 5 of the 6 study species (*A. fluviatilis*, *A. aquaticus*, *B. buceratus*, *C. pseudogracilis*, and *G. pulex*) with the eDNA method in 3 to 9 of the 14 sampling sites (Table S7). We were unable to detect *T. waeneri* with the eDNA method.

Site occupancy based on positive species detection varied among the 5 species and between the eDNA and kicknet methods (Fig. 4). *Asellus aquaticus* and *B. buceratus* could be detected at more sites with the eDNA than the kicknet method. All other species were detected more often with the kicknet than the eDNA method. The % positive equivalency, i.e., the combined positive detection success with the kicknet and eDNA method at the same site, was 56% for *A. fluviatilis*, 43% for *A. aquaticus*, 100% for *B. buceratus*, 43% for *C. pseudogracilis*, and 71% for *G. pulex*.

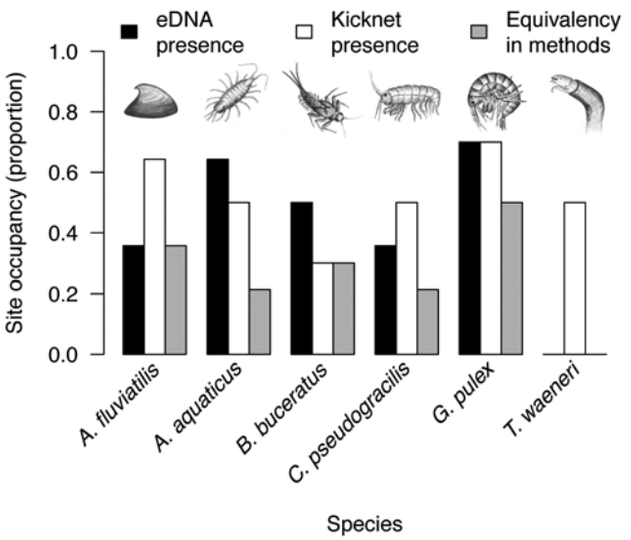

Figure 4. Proportion of sites occupied based on the environmental DNA (eDNA) and kicknet methods, and equivalency between methods in detecting different macroinvertebrate species. Maximum possible number of sites was 14 for species living in lakes and rivers *(Ancylus fluviatilis*, *Asellus aquaticus*, *Crangonyx pseudogracilis*, and *Tinodes waeneri*) and 10 for species living in the study area in rivers only (*Baetis buceratus* and *Gammarus pulex).* Black bars show the proportion of sites in which a species was detected based on the eDNA method. White bars show proportion of sites in which a species was detected through kicknet sampling. Gray bars denote equivalency in both methods, i.e., positive detections at the same sampling sites by both the eDNA and the kicknet method.

DISCUSSION

eDNA as a novel, present-day species detection method has been applied in aquatic systems mostly to amphibians or fish species (e.g., Ficetola et al. 2008, Dejean et al. 2011). Thomsen et al. (2011) and Goldberg et al. (2013) applied it to a few invertebrate species in rivers or ponds, respectively. We expanded the range of macroinvertebrate detections and were able to detect 5 of 6 macroinvertebrate species (2 amphipods, an isopod, a limpet, and a mayfly) in lentic and lotic systems (Fig. 4, Table S7). The detection rate for the mayfly species *B. buceratus* was equivalent for both methods. Thus, we were able to document the species' presence with the eDNA surveillance approach for all sites where the kicknet method established the species' presence. The isopod *A. aquaticus* was detected more often with the eDNA than the kicknet method. However, only ⅓ of the detections were congruent with presence found by the kicknet method. This result indicates some false-negative detection for the eDNA method and might also indicate a high rate of false negatives for the kicknet method, a point that has been discussed extensively (e.g., Barbour et al. 1999). Three more species were detected with both approaches (*A. fluviatilis*, *C. pseudogracilis*, and *G. pulex*), but these species were detected more often with the kicknet than with the eDNA method. All of these species are used as indicator species in water-quality and biomonitoring assessments (e.g., Stucki 2010), are nonnative species (*C. pseudogracilis*), or are listed as vulnerable species (*B. buceratus*) whose occurrence must be monitored at low population densities. Our work shows that the eDNA method has potential utility for surveying occurrence of macroinvertebrates in aquatic systems.

We compared the findings of the eDNA method directly with standardized and simultaneously applied conventional kicknet sampling (Barbour et al. 1999, Stucki 2010). Comparisons of conventional and eDNA surveillance methods already have been made (e.g., Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2011, Olson et al. 2012), but methods were rarely applied simultaneously or were focused on 1 species only (Goldberg et al. 2013, Pilliod et al. 2013). We found that the taxon-specific match of the 2 methods was moderate to high (Fig. 4, Table S7), but we also found differences (Thomsen et al. 2011, Pilliod et al. 2013). For some species, the eDNA method was as good as or better than the kicknet method for detecting a species' presence (Fig. 4), whereas for other species, the kicknet method demonstrated the presence of individuals that were not detected with the eDNA approach. Our results for *B. buceratus* show the possible advantage of eDNA over the kicknet method as a surveillance tool. We detected the species with the eDNA method at all sites where it was found in the kicknet samples, and we detected it at 2 sites where it was not detected by the kicknet method. *Baetis buceratus* can occur at low density (Lubini et al. 2012), which

makes it difficult to detect by kicknet methods. Furthermore, up to 60% of all *Baetis* species in macroinvertebrate kicknet samples are overlooked by experts in the sorting process (Haase et al. 2010). Jerde et al. (2011) showed that eDNA surveillance is potentially more sensitive than conventional methods for invasion fronts of Asian carps, and we suggest that the same is true for populations of native invertebrates that occur at low densities and are often overlooked in kicknet samples.

High equivalency between the kicknet and eDNA methods may be possible for all species with further optimizations of eDNA sampling techniques in aquatic systems and by taking into account species-specific aspects of biology and possibly abundance. However, we advocate a conservative and well-grounded approach to prevent wrong expectations in the rapidly changing field of eDNA. False negatives arose for 5 of the 6 species with the eDNA approach (Fig. 4). They can occur for reasons that reflect the limitations of eDNA as a surveillance method (Schmidt et al. 2013). Our sensitivity test showed that the designed primers have detection limits at low DNA concentrations. We extracted total DNA from tissues and measured double-stranded DNA (dsDNA), which yields upper-end estimates of DNA concentration because only some of this DNA is from our target gene COI. Thus, the real copy number of COI in our experiment probably is (much) lower than the concentrations reported.

Wilcox et al. (2013) stated that primer specificity is important for successful detections, a statement with which we completely agree. However, low specificity is not necessarily the (only) source of low detection rates. Primer-BLAST showed high species specificity for the primer pair of *T. waeneri* (Table S4), and we used an optimized PCR protocol, but we were unable to detect this species with eDNA despite its detection via the kicknet method. Our inability to amplify eDNA for this species suggests that other variables affect eDNA detection. A possible explanation lies in the biology of the species. *Tinodes waeneri* is a caddisfly and lives in a sand case, as is typical of most caddisflies. The case might reduce or delay the release of DNA from this species, eventually limiting the ability of the eDNA method to detect it. In contrast, we had a relatively unspecific primer pair for *G. pulex*, but still found that 88.8% of all sequenced amplicons were positively identified as *G. pulex*. The quality of the remaining 11.2% of sequences was too low to align them with specific species in GenBank. Furthermore, the equality between kicknet and eDNA methods was 71.4% for *G. pulex*, indicating that the eDNA method was able to detect the species at a level comparable to the kicknet method. Our data also showed that a nonspecific primer can lead to good results. However, sequencing all amplicons is a conservative approach, and we recommend that future users of primer pairs designed here continue to use sequence confirmation to avoid false positives in other regions. Combined, the detection results for the species in our study indicate that high specificity of the primer pair should be the aim, but does not guarantee the success of the method. In general, species-specificity of primers tested against sequence databases, such as GenBank, does not guarantee that primers will be specific in the field, especially when sequence data for many (closely related) species are missing from databases, such as GenBank. In such a case and depending on the study questions, we advocate sequencing all amplicons as a necessary precaution that should be required for determining true detections in eDNA studies.

False negatives also can occur with the eDNA method because of incomplete sampling. We first used a water volume of 280 mL, and then analyzed another 900 mL. This increased volume resulted in more detections, especially for *A. aquaticus* (Table S7). However, it also increased the risk of false positives. Most of our sampling sites were in lotic systems, and target DNA probably is lost by downstream flow or diluted by other sources of water. Furthermore, lotic systems are shallow and well mixed, and these factors probably increase the rate of degradation of eDNA by ultraviolet light and mechanical breakdown relative to in lentic waters (Wilcox et al. 2013, Barnes et al. 2014). This reduction of eDNA probably means that larger volumes of water must be sampled in lotic than in lentic systems to increase detection, an approach that was successful for *A. aquaticus* (Table S7). However, sampling larger volumes of water comes with the risk of collecting DNA that has been contributed from populations at further upstream sites, although the species is not locally present (Deiner and Altermatt 2014).

CONCLUSIONS

Environmental DNA as a surveillance method is in a development phase (Lodge et al. 2012, Taberlet et al. 2012), and many open questions exist about factors that influence species detection by an eDNA approach. We showed that relatively good agreement between results with eDNA and kicknet samples could be achieved for a set of macroinvertebrate species used in biomonitoring and that may occur in low densities. The eDNA method detected 2 species (*B. buceratus* or *A. aquaticus*; Fig. 4) equally or better than kicknet sampling, 3 species less well than kicknet sampling, and failed to detect 1 species. We see high potential for the eDNA method in monitoring aquatic diversity. At present, it can complement but not replace existing monitoring strategies. However, eDNA has been used to monitor invertebrates in aquatic systems only in the last 2 to 4 y and techniques are improving rapidly, whereas the kicknet method is well established and few optimizations are likely to be made (Barbour et al. 1999). Most important, the eDNA method may be able to deliver data on a much finer time scale than the kicknet method, which is feasible only at spe-

cific times of the year and depends on larval development for many species. For example, the eDNA method could be incorporated into monthly or weekly sampling regimes already implemented for monitoring water chemistry in our study area and in many other places (AWEL 2012). The kicknet method may still be better than the eDNA method for making abundance estimates or proving the local presence of a species. For example, many biomonitoring programs are based on abundance data (e.g., Species at Risk [SPEAR] index), and it is unclear whether eDNA can realistically supply abundance estimates. Moreover, given the potential of eDNA to drift from upstream sites, verification of the presence of a local population at a particular location may still require sampling of individuals (Jerde et al. 2011). We suggest complementary use of the 2 methods to take advantage of their individual strengths.

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Data Accessibility

The final DNA sequence assembly (Table S6) and eDNA sequences that could not be identified through sequence databases (Table S8) were uploaded as online supplemental material.

Author contributions

EM, KD, and FA designed the study. EM and KD developed and performed the work on macroinvertebrate detection using eDNA. PS performed and analyzed conventional kicknet samples. EM, KD, and FA analyzed data and wrote the paper.

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