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Fishing in the Water: Effect of Sampled Water Volume on Environmental DNA-Based Detection of Macroinvertebrates

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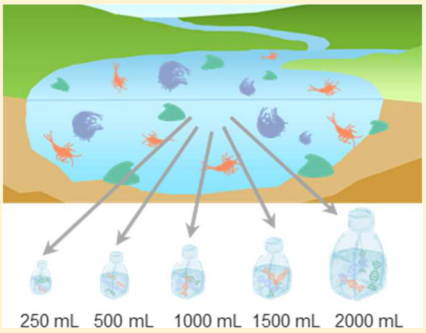
Supporting Information

ABSTRACT: Accurate detection of organisms is crucial for the effective management of threatened and invasive species because false detections directly affect the implementation of management actions. The use of environmental DNA (eDNA) as a species detection tool is in a rapid development stage; however, concerns about accurate detections using eDNA have been raised. We evaluated the effect of sampled water volume (0.25 to 2 L) on the detection rate for three macroinvertebrate species. Additionally, we tested (depending on the sampled water volume) what amount of total extracted DNA should be screened to reduce uncertainty in detections. We found that all three species were detected in all volumes of water. Surprisingly, however, only one species had a positive relationship between an increased sample volume and an increase in the detection rate. We conclude that the optimal sample volume might depend on the species−habitat combination and should be tested for the system where management actions are warranted. Nevertheless, we minimally recommend sampling water volumes of 1 L and screening at least 14 μL of extracted eDNA for each sample to reduce uncertainty in detections when studying macroinvertebrates in rivers and using our molecular workflow.



INTRODUCTION

By shedding hairs, cells, gametes, or feces, all organisms leave traces of their occurrence in the environment in the form of so-called environmental DNA (eDNA). Recent reviews illustrate that many eukaryotes, including plants and animals, are readily and noninvasively detected from traces of their DNA found in water, soil, and air.[1,2] The utility of this noninvasive molecular method for species detection has large implications for environmental management actions[3] and subsequent policy and stakeholder decisions, e.g., as in ref 4.

The method of detecting macro-eukaryotic species from traces of their DNA in the environment is, however, in a rapid development phase.[1,3] Experimental evidence of the power, as well as the limitations of the tool, is greatly needed to effectively track organisms in their environment from eDNA. Of particular concern are false-negative detections; for example, see refs 5−7. False-negative detection, or process type II error, in terms of environmental DNA means that there is no DNA detected, although the species is present at the sampled location (please note that false-negative detections can also occur during "classic sampling"; see, for example, ref 8). False-negative detections may be of particular concern for invasive species and species threatened with extinction because not detecting the species can have severe consequences for management decisions. It is therefore paramount that we gain a better understanding of the causes of false-negative detections with eDNA, such that it can be established as a viable and defensible method for species detection.[2,9]

Reasonable work has already focused on the causes of false-negative detections and the possible means by which false negatives can be reduced. Causes of false negatives are due to the inherent problem of the detection limits of the molecular technology used to capture, extract, and amplify the DNA found in the environment,[10−13] but in the case of metabarcoding approaches, they are also due to limitations of reference databases. The latter are not discussed here further because we focus on a targeted approach; see, for example, refs 14 and 15. Far less attention, however, has been paid to estimating how sampling bias affects the detection rate for a species' eDNA and subsequently results in false-negative detections. Any biological sample suffers from a sampling bias based on the probability of detection and is governed by the method(s) used for detection.[16] Environmental DNA detection is no exception and has mainly two steps, which can cause a sampling bias either in the field or in the lab.[5] The first

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Table 1. Overview on Published Methods and Quantities Used for Filtering eDNA from Macrofauna in Natural Aquatic Systems as Well as Used PCR Conditions[a]

authors	year	citation	filter material	filter pore size (μm)	sampled water volume (mL)	type of PCR	template volume used for PCR (μL)	total PCR volume (μL)	number of PCR replications	samples per site (e.g., volume replication)	total PCR replication per site	volume of extraction screened
Schill and Mathes	2008	18	polyethylensulfone	0.22	100−1000	real time	2.5	12.5	6	1	6	15
Kortbaoui et al.	2009	32	nitrocellulose	022 or 0.45	150−300	singleplex	NA	50	1	NA	NA	NA
						nested	2[c]	50	1	NA	NA	NA
Goldberg et al.	2011	19	cellulose nitrate[b]	0.45	5000−10000	standard	1	10	1	1 (10 L), 2 (5L)	1−2	1−2
			cellulose nitrate[b]	0.45	5000	multiplex	1	7	6	1	6	6
Jerde et al.	2011	33	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Minamoto et al.	2012	34	polycarbon	3	2000	standard	4	25	1	1	1	4
Olson et al.	2012	35	glass fiber	1.5	8000	standard	1	10	10	1	10	10
Takahara et al.	2012	36	cellulose acetate	3	2000	quantitative	5	20	3	1	3	9
Tambalo et al.	2012	37	NA	0.45	500	quantitative	2−4	25	2	1	2	4−8
Thomsen et al.	2012	38	nylon	0.45	500	standard	2	25	8	3	24	48
Goldberg et al.	2013	39	cellulose nitrate	0.45	4000	quantitative	2.5	10	3−9	3	9−27	22.5−67.5
Jerde et al.	2013	40	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Mahon et al.	2013	41	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Pilliod et al.	2013	42	cellulose nitrate[b]	0.45	1000	quantitative	2	10	3−6	1	3−6	6−12
Schmidt et al.	2013	9	polyethylensulfone	NA	600	quantitative	NA	NA	2	1	2	NA
Takahara et al.	2013	29	cellulose acetate	3	1000	real time	2	20	8	1	8	16
Vuong et al.	2013	43	NA	0.45	300	standard	NA	25	NA	NA	NA	NA
						nested	1[c]	50	NA	NA	NA	NA
						quantitative	5	20	3	NA	NA	NA
Wilcox et al.	2013	12	glass fiber	1.5	6000	quantitative	4	20	26−32	1	26−32	104−128
Eichmiller et al.	2014	27	glass fiber	1.5	200	quantitative	5	25	3	1	3	15
Jane et al.	2014	44	glass fiber	1.5	6000	quantitative	4	20	3	1	3	12
Keskin et al.	2014	45	polyethylensulfone	0.22	2000	standard	5	25	3−6	3	9−18	45−90
Mächler et al.	2014	21	glass fiber	0.7	900	standard	2	15	8	1	8	16
Pilliod et al.	2014	46	cellulose nitrate[b]	0.45	2000	quantitative	2	10	3	1	3−6	6−12
Amberg et al.	2015	47	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
			glass fiber	1.5	2000	quantitative	1	25	8	NA	NA	8
Fukumoto et al.	2015	48	glass fiber	0.7	4000	real time	2	20	4	1	4	8
Hunter et al.	2015	49	cellulose nitrate	0.45	250−1000	quantitative	NA	20.4	3	1−3	9−27	NA
Janosik and Johnston	2015	50	glass fiber	1.5	2000	standard	1	25	3	NA	NA	3
Laramie et al.	2015	51	cellulose nitrate	0.45	1000	quantitative	3	15	3	3	9	27
McKee et al.	2015	13	cellulose nitrate	0.45	250−1000	quantitative	3.75	15	3	1	3	11.25
Spear et al.	2015	28	cellulose nitrate[b]	0.45	1000	quantitative	3	15	3−6	1	1	9−18

[a]If dilution series were done, the given volume for the PCR template is the maximum. Samples per site are only reported if they are taken at the same sampling site, and several samples that were taken within the same sampling system were not reported. [b]Used only half a filter to extract DNA. [c]Template of first PCR product. [d]NA: not available from publication.

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sampling bias can happen when a particular amount of the environmental sample is collected, such as a set volume of air, soil, or water. The second sampling bias can happen when amplifying the targeted species' DNA from a small fraction of the total purified DNA contained in the sample. Both of these steps involve subsampling the potential pool of DNA that is tested and can result in a false-negative detection simply due to sampling error.

Focusing on freshwater, there are two possible environments to collect DNA from, namely from the sediment or from the water column. Here, we focus on estimating detection rates from water because DNA extracted from water has been commonly used in many macro-organisms' detection protocols (Table 1). Water-based samples are thought to reflect the contemporary, regional community of macroinvertebrates, while sediment-based samples are more likely to reflect local communities, possibly integrating over time. It was not the goal of our study to compare these two methods because the methods differ and are covering different, complementing aspects. For a comparison and discussion of sediment-based samples, see, for example, refs 5 and 17.

In a water-based approach, a first sampling bias can be introduced when different sets of water volumes are used. Likely, the volume of water sampled in different studies (for an overview on volumes used in previous studies using filtration, see Table 1) has been a choice of practicality based on the specific field and molecular protocol used to capture and concentrate the eDNA (i.e., the logistical aspects of sampling) and is not necessarily a reflection of the optimal amount needed to reduce uncertainty in the detection rate. For example, when eDNA is captured from freshwater through precipitation, usually 15 mL of water is used due to the limitation of the centrifuge size needed for the next step in processing the water in most standard molecular laboratories. Filtration from freshwater as a capture method is more flexible with respect to volume, and previous studies have thus used volumes ranging from 100 mL[18] up to 10 L,[19] with an average of approximately 2 L (Table 1). Thus, when left to interpretation and method choice, one could justify to sample and filter 100 mL to 10 L, but it is unclear if and how the volume of water sampled affects the detection rate for a species.

A second sampling bias can occur at the polymerase chain reaction (PCR) stage, where a wide range of total volume of extracted eDNA screened for targeted DNA has been used (Table 1). This is further confounded by the fact that varying molecular protocols have been used for the purification of DNA from freshwater, such that the total DNA screened in addition to the total eDNA recovered from the sampled water is also a possible confounding factor.[10] Surprisingly, we have no clear evidence pointing to an optimal amount of extracted eDNA needed to reduce false-negative detections (Table 1). For example, Goldberg et al.[19] screened 1 μL (but extracted DNA from 10 L), while Wilcox et al.[12] screened 128 μL of their DNA extraction (Table 1). It is known that the PCR has an inherent stochastic component, which plays a major role when DNA concentrations are low. Because this is typical for extracted DNA from environmental samples, the stochastic component of PCR needs to be considered when performing PCR on eDNA. Overall, it is not completely clear how much volume of the total extracted DNA should be screened to have a precise estimate of the effect on the detection rate for a species.

In this study, we tested the effect of sampling at these two stages and how it affects false-negative detections. Specifically, we sampled DNA from the environment in different volumes of water and then analyzed different volumes of DNA extracted from a given amount of water. We sampled independent volumes of water ranging from 250 to 2000 mL and tested for the detection of three macroinvertebrate species belonging to the orders of Mollusca, Ephemeroptera, and Amphipoda at a location in a river where all three species are known to be present. We compared the detection rate with respect to the volume of sampled water and the volume of extracted DNA that was screened. We conclude with recommendations for optimal volumes of water to sample and how much volume of extracted DNA to screen to reduce false-negative detections. Our recommendations refer to a similar set of species and study systems (i.e., macroinvertebrates in rivers). For other species or habitats, they may be used as first guiding values.

MATERIAL AND METHODS

Field Sampling. Our study site was located at the river Glatt (47°26′35.21″ N, 8°33′03.94″ E). It is a natural river belonging to the headwaters of the river Rhine catchment in Switzerland (for pictures of the study river, see ref 20). We sampled water on September 17th and 30th of 2014. These sampling dates reflect the classic sampling time points for macroinvertebrate study (commonly done either in spring or fall). We sampled at two time points to avoid spurious effects due to a given day's hydrological regime and that were close enough to each other to avoid changes in the communities researched. On each day, we sampled two replicates of each of the following volumes: 250, 500, 1000, 1500, and 2000 mL. This range of volumes was chosen based on previous work (see Table 1) to be suitable for our habitat (that is, freshwater streams and macroinvertebrates) and is reflective of most of the previous volumes considered. We sampled each volume independently in one or two individual 1 L sterile octagonal polyethylene terephthalate bottles (VWR International, Radnor, Pennsylvania) that were previously decontaminated with 10% household bleach, rinsed with Milli-Q (Merck Millipore, EMD Millipore Co., Billerica, Massachusetts) water and exposed to ultraviolet C light (UVC) and sealed in a DNA clean lab to remove all possible contaminants of DNA. We collected surface water from the edge of the river and filtered each volume on site. For each of the sampled volumes, we sequentially filtered batches of 250 mL of water onto a single 25 mm 0.70 μm glass-fiber filter (GF/F, Whatman International Ltd., Maidstone, U.K.). The total number of filters used for each volume class ranged from one to eight filters. The filters were housed in a 25 mm filter case (Swinnex, EMD Millipore Co., Billerica, Massachusetts) that was attached to a disposable 50 mL syringe. For each volume class, we used the same filter housing and the same syringe and changed only the filter as necessary to process the total volume. After filtration, we transferred the filters into individual 1.5 mL tubes containing tissue lysis buffer (100 mM Tris−HCL pH 8.0, 5 mM EDTA pH 8.0, and 0.2% SDS, 200 mM $NaCl_2$) using tweezers that were decontaminated with 10% household beach between volume replicates and rinsed with ethanol. The tubes were immediately stored on ice. The time between the first and last sample was approximately 2.5 h during field filtration. Samples were immediately transported to the laboratory with a maximum travel time of 30 min. Additionally, we created two negative filtration controls, which consisted of decontaminated Milli-Q water treated with UVC light and sealed in a DNA-free laboratory. We brought this water to the field and filtered 2000

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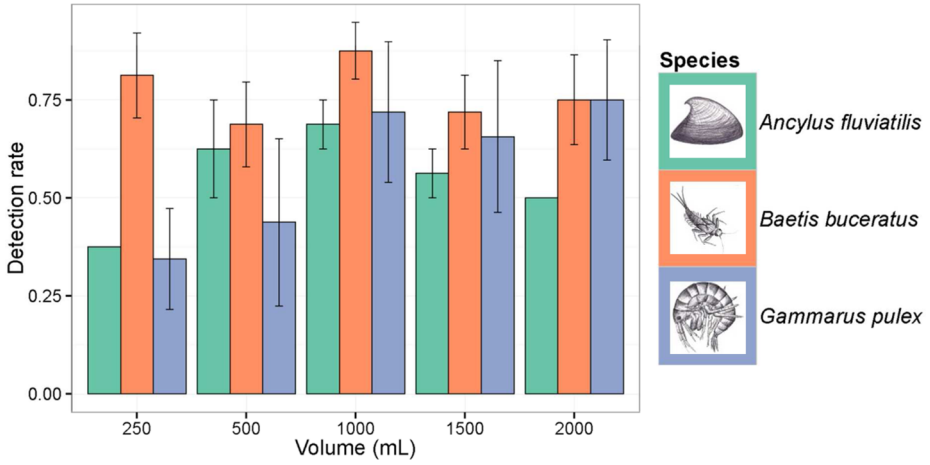

Figure 1. Detection rate, as a proportion of positive amplifications across eight PCR replicates for each volume replication, relative to water volume sampled. Error bars stand for standard errors across four samples (*B. buceratus* and *G. pulex*) and two samples (*A. fluviatilis*), respectively.

mL before environmental samples were taken on each sampling day, resulting in four negative filtration controls. Upon return to the laboratory, we immediately began the extraction as described below.

DNA Extraction and Species eDNA Amplification. In the laboratory, we performed a modified cell lysis phenol−chloroform−isoamyl extraction on each single filter, as this has been shown as an effective extraction method for eDNA from glass-fiber filters.[10] We added for each set of extraction a negative control (hence called the negative extraction control). All eDNA extractions of each target volume were subsequently pooled, such that eDNA was resuspended across all filtration volumes in a total volume of 100 μL. For example, the two filters used for the 500 mL volume were each resuspended in 50 μL and then pooled to equal the total extracted DNA volume of 100 μL. All pooled DNA extractions were cleaned with the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, California) according to the provided protocol. To countervail the volume loss during cleanup, we added 50 μL of AE buffer (this is the elution buffer from the DNeasy Blood and Tissue kit delivered from Qiagen GmbH, Hilden, Germany) to each cleaned DNA extraction to allow for testing of multiple species with the same extraction. Cleaning eDNA with this additional step has been shown to be effective for the removal of PCR inhibition of riverine samples of environmental DNA,[13] and we did not want PCR inhibition to additionally confound the detection rate. All pooled and cleaned extractions were quantified by using the Qubit (1.0) fluorometer following the recommended protocol for the high-sensitivity (HS) assay for dsDNA (Life Technologies, Carlsbad, CA) and can be consulted in Figure S1.

We conducted standard PCR because the primers used in this study were designed for standard PCR for a previous study[21] and thus allows comparability. Additionally, we wanted to use a method that seems commonly available to federal offices. We then performed eight PCR replicates in which each replicate screened 2 μL with concentrations ranging from 0.153 to 3.73 μg/mL of extracted DNA, equaling 16 μL screened for each of the following three target species: *Ancylus fluviatilis* (Mollusca), *Baetis buceratus* (Ephemeroptera) and *Gammarus pulex* (Amphipoda). On the basis of previous studies, we know that these species belong to the regional species pool at this site from long-term monitoring data (1995 to 2012) provided by the Canton of Zurich and our own sampling.[22−24] All negative filtration controls and all negative extraction controls were tested individually in eight PCR replicates for the presence of each species. We used primer probes previously designed and tested for eDNA detection of these species at the study site.[21] For each PCR run, we added a negative PCR control by adding molecular grade DNA-free water (Sigma-Aldrich, Co. LLC. St. Lewis, MO) as a template and a positive PCR control (using tissue-extracted DNA from the target species as PCR template). The PCR components and thermocycling temperature were performed exactly as described in Mächler et al.[21] and can be found in Appendix I of the Supporting Information. All PCR products were visualized by electrophoresis on a 1.4% agarose gel stained with peqGreen (VWR International, Radnor, Pennsylvania).

From each volume of water, we confirmed at least one positive PCR reaction by using Sanger sequencing. We cleaned the PCR product with Exo I nuclease (EXO I) and shrimp alkaline phosphatase (SAP) (Thermo Fisher Scientific, Waltham, Maryland) as described in Mächler et al.[21] Sequencing was performed in both directions with BigDye Terminator (version 3.1) system on an ABI 3730xl. The software Sequencher version 4.9 (Gene Codes, Ann Arbor, Michigan) was used to align, edit, and compare our sequences with previous eDNA sequences obtained from this site in a previous year and from tissue-derived sequences.[21] We used the same criteria for a positive detection (band present on a gel and sequence confirmation for each experimental volume) and rigorous laboratory precautions as described in Mächler et al.[21] by creating, in addition to our negative filtration controls, negative controls for extraction and PCR. In total, we screened 4 negative filtration, 2 negative extraction, and 12 negative PCR controls for potential contamination.

Analysis. We analyzed the detection rate of each individual species with generalized linear models (GLMM). Volume of water was used as the predictor variable and detection rate as a binary response variable consisting of the number of positive and negative detections out of the eight PCR replications. We used the two replicates per volume on a single day as a random effect, nested within the sampling day for *G. pulex* and *B. buceratus*. For *A. fluviatilis*, we had only one sampling day due to a contamination on the first day.

We tested how the uncertainty in detection rates changed as a result of increasing the amount of extracted DNA screened for each species using a resampling approach (bootstrap

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approach) of the individual assessments. We sampled 10 000 outcomes in detection rates from our data when screening 2−16 μL of DNA. As a measure of uncertainty, we subsequently calculated the median range (absolute difference between minimum and maximum) in detection rates over all outcomes for a given sample volume and species (data from the two sampling days were pooled). When uncertainty is 1, detection rates can be any value between 0 and 1, that is, the estimate is uninformative; when uncertainty is 0, the detection rate is the same for all outcomes and is maximally informative. All statistical analysis were done in R version 2.15.3 (R Development Core Team 2014)[25] and the package "lme4"[26].

RESULTS

All three species were detected at the sampling site by the use of eDNA in all volumes and on both sampling dates (Figure 1). We found a positive, significant relationship between sampling volume and detection rate for *G. pulex* ($p < 0.05$, Table 2). For the other two species there was no significant relationship (*A. fluviatilis* $p = 0.78$, *B. buceratus* $p = 0.72$; Table 2).

Table 2. GLMM Results on the Effect of Volume for the Detection of Each Species

	coefficient	standard error	*Z* value	*p* value
(A) *Ancylus fluviatilis*				
intercept	0.0973	0.4309	0.23	0.82
volume	0.0001	0.0004	0.28	0.78
(B) *Baetis buceratus*				
intercept	1.5214	0.7362	2.07	0.04
volume	−0.0001	0.0003	−0.36	0.72
(C) *Gammarus pulex*				
intercept	−1.1762	1.1871	−0.99	0.32
volume	0.0016	0.0004	4.34	<0.01

We showed for all three species that the uncertainty in the detection rate decreases when increasing the volume of extracted eDNA screened (Figure 2). The uncertainty decreased differently between the three species; however, all species reached 0 uncertainty (i.e., detection rate never changes between outcomes) when screening at least 14 μL of extracted DNA.

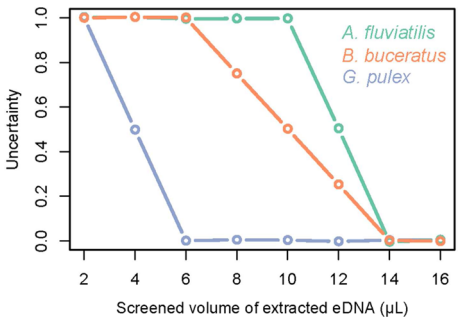

Figure 2. Uncertainty in the detection rate relative to the screened volume of extracted environmental DNA. When uncertainty is 1, detection rates and thus the outcome vary between 0 and 1; when uncertainty is 0, detection rate is completely consistent between outcomes. The uncertainty of the detection rate is decreasing with increasing volume of screened DNA for all three species and reaches 0 at 14 μL of screened volume of eDNA.

We detected a contamination (a confirmed amplicon of the targeted species) in one negative filtration control for *A. fluviatilis* on the first sampling day. From the total 16 μL of extracted DNA, the negative filtration control screened showed a positive amplicon in three PCR replicates (i.e., 6 μL of screened DNA). Attempts to measure DNA concentration from this negative filtration control failed because DNA concentrations were too low (limit of detection reported for Qubit high-sensitivity assays was <0.001 μg/mL). All other negative controls (filtration, extraction, and PCR) were blank. The three positive amplifications of this negative filtration control happened within replicates in the same PCR set up, and thus we are confident that the contamination happened during the preparation of the PCR reactions and was not a contamination from the field. Unfortunately, due to the testing of the extractions for so many replicates and species, we ran out of the extracted DNA before being able to repeat this PCR. To be most conservative, we excluded the data for this species from the first day from all analyses.

DISCUSSION

Comparing the effect of sampled water volume and an eDNA-based detection across three macroinvertebrate species, we surprisingly found that only one species (*G. pulex*) had a positive relationship with an increased detection rate when more water was sampled. The detection rate of the other two species did not correlate with sampled water volume. Based on the results of a previous study using the same primers,[21] we calculated the detection probability (i.e., the detection with eDNA divided by the proven presence with traditional monitoring method) in river systems using the same primers. This detection probability was 0.83 for *A. fluviatilis*, 1 for *B. buceratus*, and 0.71 for *G. pulex*. Thus, as the latter species seems to have in general a lower detectability, it may be the one most affected by the total amount of water volume sampled. Indeed, we found a dependence of water volume and detection rate for this species at the lower volumes, but the effect leveled off above 1 L and then was saturated at the species' overall detection probability (i.e., the detection rate at 1 L was 0.72 ± 0.18 and at 2 L was 0.75 ± 0.15). Surprisingly, we did not find a positive relationship between water volume and detection rate for all three species. We speculate that such a relationship exists also for the other two species. We think that we did not reach the lower limit of the detection where this relationship exists for *A. fluviatilis* and *B. buceratus*, while for *G. pulex*, we were within the water volume range where the saturation occurs. Our results thus indicate that detection rates may vary by species and volume, which should be considered when designing targeted eDNA detection tool.

All species' DNA was detected in at least one PCR replicate at the smallest volume of 250 mL. The detection rate had a lower uncertainty at higher volumes of DNA extraction screened compared to lower volumes (Figure 2) and indicates that screening more of the DNA extraction allows for a smaller uncertainty in detection rate for a species with the used protocols across any volume. The volume of extraction we screened is similar to other studies using filtration as the DNA-capturing approach,[27−29] and these studies sampled similar volumes (0.2 to 1 L) of water (Table 1). Even when we sampled 2 L of water, we found in none of the three species studied a positive signal in 100% of the PCR replications. PCR is a stochastic process, and subsequently, PCR replication (e.g., the volume of screened eDNA) cannot be neglected even when

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sampling larger volumes. In Figure 2, we illustrate the importance of screening enough volume of extracted DNA to decrease the uncertainty in the outcome of the detection. Although this is intuitive, it is an important aspect to keep in mind when creating a protocol for species detection with eDNA. We recommend testing volume dependence for each species that should be detected with eDNA so that eDNA protocols are optimized with respect to sampling volume and screened volume of eDNA extractions. When resources are limited, we suggest maximizing the volume of extracted eDNA, which is screened and not necessarily to maximize the water volume sampled. PCR replication is more cost-effective than the filtration and extraction of larger volumes of water due to two reasons: first, at our study site we were not able to filter more than 250 mL on one single glass-fiber filter because of free-floating particles that clog the filters, even though we conducted our work in a river that is known to have an overall low level of particle and sediment load. For filtering volumes of 2 L, we needed up to eight filters. The usage of multiple filters for the same sample is a time-consuming and costly step, as each filter costs about 1 USD. Second, the extraction will be even more time-intensive, as each of the filters needs to be extracted separately due to limitations of tube sizes and a limited ability to handle large volumes (greater than 2 L) for most standard molecular genetic laboratory centrifuges. One may need to validate these results across various river systems to adjust methods for different eDNA quality and degradation, primer sensitivity, and sediment loads. However, the conditions in the river Glatt are likely valid for rivers and streams in human-modified temperate landscapes with a mixed land-use of urban areas, forests, and agricultural land-use.

We especially want to highlight the relevance of negative controls. We performed negative controls during three steps in the handling process: filtration, extraction, and PCR. Contaminations can occur, especially while filtering in the field, but through a good study design, it is possible to track down the source of contaminations. We suggest that it is relevant to not only screen a certain volume of extracted DNA but also to screen an adequate volume for the negative filtration controls. In our study, we had contamination for one species in one filtration control on the first day. Because all of the positive amplicons showed up in one set of PCR, it is very likely that our contamination happened during the preparation of the PCR. However, because we cannot rule out field contamination, we were stringent and have excluded all detections of this species from the first day. The implication of excluding dubious data is especially important for environmental DNA work, in which low amounts of DNA are handled, and small contaminations can lead to false positives. Given our findings regarding the effect of volume (either from the environment or from the extraction) on uncertainty in detection rates, we especially encourage eDNA researchers to report the number and volume of negative controls that are screened to ensure accountability. When this tool becomes used in controversial cases, it will be paramount to upholding the same practices and standards we apply to samples to negative controls as well for proper inference.

Optimization of single-species detection through eDNA may depend on the specific-species and environmental settings; however, some critical considerations and guidelines can still be inferred from our results. Overall, we find that there may be different factors contributing to the successful detection of species using eDNA. First, we conclude (on the basis of first principles) that at a given concentration of eDNA molecules in the environment, there must be a lower volume threshold at which detection becomes less likely, while the detection rate saturates at higher volume due to other factors (e.g., primer performance[12] and competition of target DNA versus nontarget DNA during PCR[21]). Our data, however, suggest that this level is below 250 mL of water, at least for the three species tested here and under this molecular workflow. Although increasing sampling volume may indeed be beneficial in reducing false-negative detections (i.e., reducing uncertainty in the detection rate in our study) for some species, increasing the volume of extracted DNA screened and primer performance may become more important due to a lower detection rate for smaller environmental sampling volumes. Second, when comparing methods, one needs to quantify not only the detection thresholds and rates of false negatives for the eDNA method but also the method it is compared with. Although the issue of both false positives and false-negative detections has been raised for eDNA approaches,[2,9] it is often ignored for traditional methods, where a perfect sampling is often implicitly assumed (see refs 8, 30, and 31 for extensive discussion on this topic). On the basis of our results, we can give a rough recommendation that sampling at least 1 L and screening a minimum of 14 μL of total extracted DNA should reduce false-negative detections, particularly for macroinvertebrates in freshwater and potentially for other macrospecies surveillance. The recommendations should be taken with some precautions because results might be changing, even within similar species and environments. We caution researchers to carefully plan sampling designs with regard to volume and encourage pilot-testing these parameters to maximize the potential detection rates for other systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b04188.

Additional information on the PCR mixture, primer and temperature regime. Figure showing eDNA concentrations relative to used sample volumes. (PDF)

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Notes

The authors declare no competing financial interest.

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