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ARTICLE

Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples

David S. Pilliod, Caren S. Goldberg, Robert S. Arkle, and Lisette P. Waits

Abstract: Environmental DNA (eDNA) methods for detecting aquatic species are advancing rapidly, but with little evaluation of field protocols or precision of resulting estimates. We compared sampling results from traditional field methods with eDNA methods for two amphibians in 13 streams in central Idaho, USA. We also evaluated three water collection protocols and the influence of sampling location, time of day, and distance from animals on eDNA concentration in the water. We found no difference in detection or amount of eDNA among water collection protocols. eDNA methods had slightly higher detection rates than traditional field methods, particularly when species occurred at low densities. eDNA concentration was positively related to field-measured density, biomass, and proportion of transects occupied. Precision of eDNA-based abundance estimates increased with the amount of eDNA in the water and the number of replicate subsamples collected. eDNA concentration did not vary significantly with sample location in the stream, time of day, or distance downstream from animals. Our results further advance the implementation of eDNA methods for monitoring aquatic vertebrates in stream habitats.

Résumé : Si les méthodes de détection des espèces aquatiques reposant sur l'ADN environnemental (ADNe) évoluent rapidement, l'évaluation des protocoles de terrain et de la précision des estimations en découlant demeure limitée. Nous avons comparé les résultats d'échantillonnage à l'aide de méthodes de terrain traditionnelles aux résultats de méthodes reposant sur l'ADNe pour deux espèces d'amphibiens dans 13 cours d'eau du centre de l'Idaho (États-Unis). Nous avons également évalué trois protocoles de prélèvement d'eau et l'influence du lieu de prélèvement, de l'heure du jour et de la distance par rapport aux animaux sur la concentration d'ADNe dans l'eau. Nous n'avons noté aucune différence sur le plan de la détection ou de la quantité d'ADNe entre les différents protocoles de prélèvement d'eau. Les méthodes reposant sur l'ADNe présentaient des taux de détection légèrement plus élevés que les méthodes de terrain traditionnelles, particulièrement quand la densité des espèces était faible. La concentration d'ADNe était positivement reliée à la densité, la biomasse et la proportion de transects occupés mesurées sur le terrain. Si la précision des estimations de l'abondance basées sur l'ADNe augmentait parallèlement à la quantité d'ADNe dans l'eau et au nombre de sous-échantillons répétés prélevés, la concentration d'ADNe ne variait pas significativement en fonction du lieu de prélèvement dans le cours d'eau, de l'heure du jour ou de la distance vers l'aval par rapport aux animaux. Nos résultats constituent une avancée dans l'application des méthodes reposant sur l'ADNe à la surveillance des vertébrés aquatiques dans les habitats lotiques. [Traduit par la Rédaction]

Introduction

Biologists need sampling tools and protocols that are designed to detect rare, secretive, at-risk, and invasive species with high levels of certainty, low cost, and minimum stress for the animals. Detection of many freshwater species, especially those that are secretive or occur at low densities, is difficult using traditional methods such as electrofishing, netting, and snorkeling (e.g., Snyder 2003; Albanese et al. 2011). Low rates of detection greatly increase field survey costs, reduce certainty of occupancy estimates, and limit inference about the distribution and status of species. Rare species or species at risk of extinction may also have sampling restrictions that prohibit trapping or handling.

The collection and analysis of environmental DNA (eDNA; DNA that has detached from the individual and is in the environment) is an emerging conservation tool that shows promise for improving detection of freshwater species (Thomsen et al. 2012; Taberlet et al. 2012). The method has been used successfully for amphibians (Ficetola et al. 2008; Goldberg et al. 2011; Dejean et al. 2011, 2012; Olson et al. 2012; Thomsen et al. 2012), fish (Jerde et al. 2011; Dejean et al. 2011; Takahara et al. 2012; Thomsen et al. 2012), insect larvae and crustaceans (Thomsen et al. 2012), and mammals (Thomsen et al. 2012). The technique works even when animals are at low densities in the wild and regardless of whether the water is standing or flowing (Ficetola et al. 2008; Goldberg et al. 2011). The rapid emergence of eDNA for species detection has generated interest among fisheries and other natural resource managers seeking cost-effective tools for inventory and monitoring of species.

As interest in applications of eDNA grows, the need for information about how field protocols and environmental conditions influence detection is apparent (Lodge et al. 2012). No standard operating procedures or protocols for field methods have been published to date, and few studies have examined the precision of different field methods. To our knowledge, four field methods have been used, each with differences in sample collection, preservation, and extraction protocols (see Pilliod et al. 2013 for details). We expect field protocols will continue to be developed, tested, and modified for specific habitat types and target species.

Before eDNA methods become widely adopted for survey and monitoring of aquatic species, practitioners will need to know how eDNA-based estimates of species occupancy and abundance relate to estimates derived from traditional field sampling

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D.S. Pilliod and R.S. Arkle. US Geological Survey, Forest and Rangeland Ecosystem Science Center, 970 Lusk Street, Boise, ID 83706, USA.
C.S. Goldberg and L.P. Waits. Fish and Wildlife Sciences, University of Idaho, Moscow, ID 83844-1136, USA.
Corresponding author: David S. Pilliod (e-mail: dpilliod@usgs.gov).

methods and how eDNA-based estimates are affected by sampling design. Recent studies have demonstrated increased detection probabilities of eDNA over traditional methods for detection of Asian carp (*Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis*; Jerde et al. 2011) and American bullfrogs (*Lithobates catesbeianus*; Dejean et al. 2012), while implementation of quantitative polymerase chain reaction (qPCR) methodologies has revealed that eDNA concentration in water samples was related to the biomass of common carp (*Cyprinus carpio*) in artificial ponds (Takahara et al. 2012) and density of common spadefoot toads (*Pelobates fuscus*) and great crested newts (*Triturus cristatus*) in natural ponds (Thomsen et al. 2012). However, spatial and temporal variability in eDNA concentration and how this variation can affect the results of eDNA studies has not yet been addressed. For example, it is unknown whether eDNA concentrations are affected by the proximity of target organisms to the location of sample collection in a water body or whether concentrations fluctuate based on the time of day when samples are collected. Finally, there have been no previous efforts to determine how the precision of eDNA-based abundance estimates is influenced by the amount of eDNA present at a given site and by the number of replicate samples collected.

The goals of this study were to (1) develop and test alternative field protocols for sampling stream water for eDNA of target species, (2) compare estimates of detection probability, density, biomass, and occupancy derived from traditional field methods with presence and amount of eDNA in water samples, (3) examine how sampling location within stream, time of day, and distance from animals influences eDNA concentrations, and (4) examine factors influencing precision of eDNA concentration estimates. Methods were optimized and evaluated for detection of Rocky Mountain tailed frogs (*Ascaphus montanus*) and Idaho giant salamanders (*Dicamptodon aterrimus*) from streams in central Idaho. These species are endemic to the Intermountain West of North America (Adams 2005; Lohman and Bury 2005). They are secretive and difficult to survey because they only breed in high-gradient streams and usually occur at low densities. Larval development of these species is slow, lasting 3 to 4 years, and some salamanders remain in streams as paedomorphic adults, which are generally indistinguishable from larvae. Tailed frog tadpoles are relatively small (mean ± SD total length: 39.2 ± 13.4 mm; mean ± SD mass: 1.0 ± 2.2 g) compared with giant salamanders (mean ± SD total length: 150.6 ± 45.0 mm; mean ± SD mass: 34.2 ± 22.0 g) in our study streams (D.S. Pilliod and R.S. Arkle, unpublished data from 2011).

Materials and methods

Stream surveys

We collected eDNA samples immediately prior to conducting traditional stream surveys at 13 streams in the South Fork Salmon River Sub-basin, Idaho, in July and August 2011 (see supplementary data, Table S1 and Fig. S1, for stream characteristics and locations[1]). We estimated detection probability, density, biomass, and proportion of transects occupied for larvae of each species by kick-netting in thirty 1 m belt transects (see Arkle and Pilliod 2010), randomly placed within a 1 km stream reach immediately upstream from our eDNA sample points. Each larva captured was weighed (wet) with a spring scale. We focused on larvae because their abundance is far greater than any other amphibian life stage in these streams. Adults are mostly terrestrial, but we occasionally observed adults and eggs in streams. We did not sample for these life stages, and they were not included in density or biomass estimates. Larval density (individuals·m^{-2}) and biomass (g·m^{-2}) were calculated for each transect and then averaged across the 30 transects per stream.

Four of the study streams were used to compare among three eDNA sample collection methods. The entire set of 13 streams was used to compare detection probabilities between eDNA (derived using the grab-and-filter sample collection method described below) and traditional methods (i.e., kick-net sampling) for each species. We also used the set of 13 streams to compare concentrations of eDNA with estimates of larval density, biomass, and proportion of transects occupied.

To ensure that kick-netting provided an accurate estimate of giant salamander abundance, we compared kick-netting and electrofishing density estimates in four streams. We used a backpack electrofisher to survey 500 m of stream immediately upstream from our eDNA sample points following a protocol developed by Cossel et al. (2012). Giant salamander density was estimated by dividing the number of larvae (and possibly paedomorphic adults) captured during electrofishing by the total area searched. Electrofishing density was a good predictor of salamander density measured by kick-netting ($n = 4$ streams, $r^2 = 0.96$, $F_{[1,2]} = 51.8$, $P = 0.019$). Hence, we were confident in using kick-net density estimates for giant salamanders in subsequent analyses.

In addition to the 13 streams described above, we conducted additional sampling designed to answer specific questions (objective 3) in two streams (Deadwood River and the East Fork of Deadwood River) near their headwaters in the Payette River Sub-basin and one stream (Weir Creek) in the Lochsa River Sub-basin, Idaho. In both forks of Deadwood River, we measured eDNA concentrations (using the in-stream method described below) of tailed frogs over a 48 h period in two reaches located approximately 50 m apart. This allowed us to examine eDNA concentrations relative to sampling location (i.e., stream reach) and time of day. We selected these streams because tailed frogs and tailed frog eDNA had been detected in them previously. Idaho giant salamanders are not present in this drainage, precluding them from these analyses. In Weir Creek, we collected eDNA samples just prior to the beginning of a 3-day, multiple-pass removal study of giant salamanders. On the first day of the study, we collected an eDNA sample, using the in-stream filtering technique, at approximately 50 m intervals in two reaches within a 2 km length of stream. Immediately after collecting eDNA samples we electrofished the entire 2 km stream reach once per day for 3 consecutive days using a protocol developed by Cossel et al. (2012). All salamanders were captured, weighed, and held until the end of the third day. The exact capture location within the stream was recorded for each salamander. Using these capture data, we created a continuous distribution of salamander abundance along the 2 km stream reach. We used this information to determine whether eDNA concentrations were influenced by the abundance of animals upstream from the location where an eDNA sample was collected. No relationship between eDNA concentration and salamander abundance upstream would indicate that eDNA concentrations are not influenced by the choice of sample collection location at the metre to tens-of-metres scale, but rather that the concentration of eDNA in the water reflects upstream salamander abundance at the reach or larger scale.

As field-negative controls, we collected eDNA samples from three additional streams outside the range of these species in the Bruneau River drainage south of Bruneau, Idaho. We did not survey these streams for giant salamanders or tailed frogs because they are >100 km south of the range of the species.

Water sample collection and filtration

We collected three replicate samples (i.e., subsamples) of surface water in the thalweg of each of our 13 focal streams, in addition to a negative control. Each water sample was collected by pumping 1 L of water through a disposable filter funnel with 47 mm diameter cellulose nitrate filter paper with a 0.45 μm pore

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfas-2013-0047.

size (sensu Goldberg et al. 2011; see Pilliod et al. 2013 for illustrations). We pumped water using a peristaltic pump or a hand vacuum pump, depending on availability. The negative control was placed randomly in the sequence of replicates and was collected by filtering 1 L of store-bought distilled water using the field apparatus. We used this negative control to assess possible sources of contamination from the apparatus, filter-paper handling procedures, or subsequent laboratory procedures.

As part of developing and testing standard protocols, we first compared three field methods in four streams: direct filtration of stream water in the field (in-stream), water collected in a 1 L Nalgene polyethylene bottle followed by immediate filtration in the field (grab-and-filter), and water collected in a 1 L Nalgene bottle, stored overnight, and filtered in the laboratory the following day (grab-and-hold). The three approaches were selected because each could be useful under different field sampling conditions. For example, in remote locations, in-stream sampling prevents field technicians from having to carry multiple 1 L water samples over long distances, and samples are preserved immediately following collection. The grab-and-filter and grab-and-hold approaches were included because they could increase efficiency for field crews sampling many water bodies in a day (e.g., sampling multiple streams at road crossings). For the in-stream method, we scooped surface water directly from the stream using the filter funnel and measured 1 L of discharge water in a graduated flask. This method has the advantage that no sterilization of equipment is required. The grab-and-filter method used stream water collected in 1 L Nalgene containers. Filtration was completed within 15 min by pouring the collected water into the filter funnel apparatus as previously described. Prior to water collection, the 1 L sample containers were cleaned with 50% bleach solution, rinsed thoroughly with tap water, air dried, and sealed in the laboratory. In the field, containers were triple-rinsed with stream water prior to sample collection. For the grab-and-hold method, we collected 1 L of stream water in Nalgene containers, stored the samples in a cooler at 5 °C for 24 h, and then filtered the samples in the laboratory using the filter funnel apparatus previously described. This last method was intended to simulate a field crew collecting water samples as part of other duties, but providing those samples to a laboratory within 24 h. All negative control samples were filtered using the grab-and-filter method.

Filter paper handling and storage

We handled and stored the filter paper the same way for each sample and were careful to avoid contamination throughout the process. Using disposable nitrile gloves, we first removed the plastic funnel exposing the filter paper. The filter paper was then rolled using forceps and placed into a 2.0 mL tube filled with 95% ethanol. Forceps were cleaned by soaking in 50% bleach solution and were rinsed thoroughly with distilled water between samples. Each tube was sealed and placed in an individually labeled plastic bag. Samples were stored at room temperature until DNA extraction, in most cases within 1.5 months. Samples from four streams were stored for 6.5 months, but DNA concentrations in these samples were not lower than expected, suggesting that degradation in ethanol is likely minimal over this time period. Each filter paper was analyzed to detect eDNA from both species simultaneously.

Genetic analysis

We extracted DNA from each filter paper using the Qiashredder/DNeasy Blood & Tissue DNA extraction kit method described in Goldberg et al. (2011). Extractions were conducted in a room where no high-quality DNA or PCR products had been handled and where researchers who have been exposed to PCR product or concentrated DNA sources are required to shower and change clothing before entering. One half of each filter paper was extracted and the other half was archived as a reserve. We updated the species-specific test described in Goldberg et al. (2011), using the originally utilized cytochrome *b* data (Nielson et al. 2001; Carstens et al. 2005), to a quantitative PCR test for additional specificity, sensitivity, and quantification abilities (see supplementary data, Table S2[1], for sequences). Amplicons from each species from each of the four streams used in the test of field methods were sequenced using BigDye Terminator version 3.1 Cycle Sequence Kit (Applied Biosystems) on a 3130xl Genetic Analyzer (Applied Biosystems) to confirm specificity. These assays were designed to detect both species of *Ascaphus* and may detect other species of *Dicamptodon* (although it is not a perfect match to any of them); however, none of these congeners co-occur with the target species for this study.

We used the QuantiTect Multiplex PCR Mix (Qiagen, Inc., Gaithersburg, Maryland, USA) with recommended multiplexing concentrations and parameters on an Applied Biosystems 7500 Fast Real-Time PCR System to conduct the assay. Reaction volume was 10 μL and we included an exogenous internal positive control (Qiagen, Inc.) in each well. If the internal positive control indicated inhibition, we diluted DNA to 1/10 in water and reanalyzed the sample. We created and analyzed an extraction negative with each set of extractions and a PCR negative with each plate of qPCR. We used 2 μL of DNA extract in each reaction and ran all reactions in triplicate. If any reaction showed incomplete evidence for the presence of DNA of either species (tested positive for one or two wells), we reanalyzed the sample. If any of the wells yielded ambiguous results during a second round, we considered the sample positive, and the quantitation amount was averaged over all replicates, as recommended by Ellison et al. (2006). If zero wells amplified on the second round after one or two amplified on the first round, we required a third round to confirm the negative result. Testing negative was indicated by no exponential phase at any point during the 50 cycles. We archived extracted DNA in a −80 °C freezer after analysis to allow for future testing.

We used DNA extracted from tail clips of each target species to create a serial dilution to develop the standard curve for quantification. We chose this method over quantifying copy number because (*i*) the ecological relevance of overall DNA concentration in water samples versus number of copies of a specific mitochondrial fragment may be higher and more comparable among species, and (*ii*) in forensic-level applications such as eDNA, having concentrated product of the target fragment for an assay handled regularly in a lab greatly increases the chance that samples will become contaminated. Standard curves for all runs had $r^2 \geq 0.98$ and efficiency averaged 118% for tailed frogs and 116% for Idaho giant salamanders. We quantified the DNA concentration in the original samples using a NanoDrop spectrophotometer and used this value to estimate the amount of DNA in each water filter sample. We validated the sensitivity of this test using positive samples collected throughout the range of the two species, and we evaluated the specificity of the test using DNA from co-occurring amphibian species (*Anaxyrus boreas*, *Pseudacris sierra*, *Ambystoma macrodactylum*, *Rana luteiventris*, and *Lithobates catesbeianus*).

Data analysis

Prior to analysis, any replicate subsample whose eDNA concentration fell outside of the 95% CI for the other samples in a given stream was flagged as an outlier. To decrease the influence of individual subsamples and to provide an estimate of the effects of outliers on analyses, we present some results both including and excluding those subsamples in calculations. No sample units (i.e., streams) were excluded from analyses.

To compare eDNA concentration estimates among the three field methods, we used mixed effect models with stream as the random effect. These and other analyses were performed in SAS 9.2 (SAS Institute, Cary, North Carolina, USA), unless otherwise stated.

Table 1. The average probability of detecting (*p*) Rocky Mountain tailed frog or Idaho giant salamander eDNA from water samples collected in four streams using different field methods.

Species	In-stream	Grab-and-filter	Grab-and-hold	Species *p*
Rocky Mountain tailed frog	1	0.83	0.92	0.92
Idaho giant salamander	1	0.92	1	0.97
Method *p*	1	0.88	0.96	

Note: Detection probability *p* was estimated as the number of replicates where a species' DNA was detected divided by the number of replicates collected in each stream ($n = 3$). Detection probability for each species (Species *p*) uses the same approach, but ignores method ($n = 9$). Likewise, Method *p* ignores species ($n = 6$). See Materials and methods for description of each water collection protocol.

We compared stream-level occupancy and detection probability estimates generated from eDNA sampling with those generated from traditional field methods. Stream-level occupancy for each species was determined by the detection of a single individual during surveys or by a water sample that amplified DNA for a species.

To determine whether eDNA concentration was related to tailed frog or salamander abundance, we used general liner modeling (GLM). For these analyses, each stream was a sample unit ($n = 13$ streams), and the predictor variable (i.e., mean density, mean biomass, or proportion of transects occupied) was calculated from the 30 belt transects sampled in each stream. The response variable, mean eDNA concentration, was calculated using the three replicate subsamples collected in each stream.

Our analyses of effects of location of sample collection, time of day, and distance from animals on eDNA concentration were carried out in a similar manner, but using a subset of streams. To test the effects of location of sample collection within streams, we ran separate GLM analyses for eDNA samples collected in three streams (Deadwood, East Fork Deadwood, and Weir). In each of these three analyses, location of sample collection was a categorical predictor with two locations in each stream. We treated time of day (0900, 1000, 1200, 1500, 2100) over a 48 h period as a categorical predictor and tailed frog eDNA concentration as the response in two streams (Deadwood and East Fork Deadwood). To test for an effect of distance from animal, we performed GLM analyses on salamander eDNA concentrations from 11 samples collected throughout a 1200 m reach of one stream (Weir) versus electrofishing-based salamander abundance measured (*i*) at the location of eDNA sample collection, (*ii*) within 10, 20, 30, 40, and 50 m increments upstream of each sample, and (*iii*) within these same increments, but offset by 10 m upstream to allow for water mixing. In these analyses, electrofishing-based salamander abundance, calculated in the various ways described above, was a quantitative predictor variable.

The precision of eDNA concentration estimates was evaluated for each species by plotting the relative standard error (RSE = SE/mean) of within-stream eDNA concentration estimates against the mean eDNA concentration estimate for each stream where the species was detected. We expected that RSE values would not be constant, but would instead depend on the abundance of eDNA in a given stream as well as the amount of within-stream replication. In ecological studies, RSE values greater than 20% are generally considered high and indicate either high heterogeneity (i.e., spatial or temporal) or inadequate within-sample unit (i.e., stream) replication (McCune and Grace 2002).

Results

Comparison of eDNA water collection methods

Detection rates were high for both species and all three sample collection methods, with in-stream filtering the only collection method with perfect detection for both species (Table 1). eDNA concentrations varied among replicates within each stream, and we found little or no evidence to suggest that one water collection method captured more eDNA or more consistent amounts of eDNA (i.e., among replicates within a stream) than the others ($F_{[2,30]} = 1.23$, $P = 0.31$ for tailed frogs and $F_{[2,30]} = 0.21$, $P = 0.81$ for giant salamanders). We found that our field protocol was resistant to cross-contamination among replicates, because no target species' eDNA was detected in any of our 16 negative control samples. We also did not amplify eDNA in samples collected in three streams outside the range of these species, indicating that the protocol did not produce false-positives from non-target species. Amplicons sequenced from these samples provided the expected sequences for each species.

Comparison of detection and abundance using eDNA versus field methods

We found that eDNA methods had higher detection rates than traditional kick-net surveys across 13 streams. At the stream level, all streams were found to be occupied by tailed frogs using both methods. Giant salamanders were not found in all streams, but the eDNA method detected giant salamanders in two additional streams compared with kick-netting.

eDNA concentration of each species was positively associated with in-stream density, biomass, and proportion of transects occupied (Table 2; Fig. 1). We found the strength of these relationships increased when eDNA replicates that were identified as outliers prior to analysis were removed from the calculation of mean eDNA concentration for their respective streams (Table 2). We found stronger relationships between eDNA and field measurements for tailed frogs, the more abundant of the two species, compared with giant salamanders. All three field metrics were correlated ($r = 0.95–0.97$), but proportion of transects occupied by tailed frogs was the best predictor of eDNA concentration, followed by density and biomass (Table 2). For giant salamanders, the proportion of transects occupied and biomass were equally good predictors of eDNA concentration, whereas density was somewhat weaker. We are uncertain about the causal relationships between these measured variables and eDNA concentration, partly because there is a paucity of information on factors influencing eDNA production and degradation in freshwater environments. Despite the greater abundance of tailed frog tadpoles (on average, we captured six times more frog tadpoles than salamander larvae), their total biomass was 14 times lower than giant salamander larvae (D.S. Pilliod and R.S. Arkle, unpublished data). The paucity of streams with high density and biomass resulted in one influential stream in these regression analyses. We found that these relationships were still significant when this stream was removed from the analyses for tailed frogs, but not for giant salamanders.

Influence of sample location on eDNA concentrations

The location of eDNA sample collection within a given stream reach had no detectable effect on estimated eDNA concentration. Tailed frog eDNA samples ($n = 20$) collected in two locations in a stream were not significantly different whether tailed frogs occurred at low ($F_{[2,18]} = 1.59$, $P = 0.224$) or high density ($F_{[2,18]} = 0.03$, $P = 0.859$). In both streams, the mean eDNA concentration at the two sample collection sites differed by less than 0.001 ng·L^{-1} when within-stream replicate outliers were excluded. These findings were consistent even when samples were collected in two stream reaches separated by 450 m ($F_{[2,10]} = 2.44$, $P = 0.15$ for tailed frogs and $F_{[2,10]} = 2.14$, $P = 0.17$ for giant salamanders).

Table 2. The relationship between field-based estimates of abundance and average eDNA concentration for two species.

Species	Predictor variable[a]	No. of eDNA replicates excluded	r^2	$F_{[1,11]}$	P
Rocky Mountain tailed frog	Density	0	0.49	10.48	0.0079
		6	0.83	52.9	<0.0001
	Biomass	0	0.36	6.1	0.0311
		6	0.78	38.19	<0.0001
	Occupancy	0	0.75	33.16	0.0001
		6	0.91	102.29	<0.0001
Idaho giant salamander	Density	0	0.24	3.47	0.0890
		4	0.27	4.15	0.066
	Biomass	0	0.38	6.8	0.0245
		4	0.41	7.65	0.0184
	Occupancy	0	0.37	6.28	0.0292
		4	0.41	7.69	0.0181

Note: Reported values are based on results of regression analyses conducted using $n = 13$ streams. Average eDNA concentration for each stream was calculated using all three within-stream replicates and was also calculated excluding within-stream eDNA replicates previously identified as outliers (outside 95% CI for a given stream).

[a]Density = average larval density (individuals·m^{-2}) from 30 belt transects per stream; Biomass = average biomass (g·m^{-2}) of larvae from 30 belt transects per stream; Occupancy = proportion of 30 belt transects per stream that were occupied by larvae. For giant salamanders, "larvae" also may include paedomorphic adults.

Influence of time of day on eDNA concentrations

The time of day when eDNA samples were collected had no detectable effect on tailed frog eDNA concentration. We found that eDNA samples collected from a stream at five times between 0900 and 2100 over a 48 h period did not have significantly different eDNA concentrations whether tailed frogs occurred at low ($F_{[5,16]} = 0.41$, $P = 0.80$) or high density ($F_{[5,16]} = 1.35$, $P = 0.29$).

Influence of upstream salamander density on eDNA concentrations

Variations in salamander density over short distances upstream from locations where eDNA samples were collected had no detectable effect on eDNA concentration. Continuous field sampling of salamanders throughout a 1200 m reach indicated substantial variability in the density of salamanders throughout the stream (Fig. 2). However, eDNA concentrations from 11 samples collected throughout this reach were not positively associated with salamander abundance as measured (*i*) at the location of eDNA sample collection, (*ii*) within 10, 20, 30, 40, and 50 m increments upstream of each sample, and (*iii*) within these same increments, but offset by 10 m upstream to allow for water mixing (Table 3). Although some relationships are nearly significant at $\alpha = 0.05$, it should be noted that these relationships consisted of negative associations between eDNA concentration and salamander abundance and thus were counter to our hypothesis about the influence of upstream salamander density on downstream eDNA concentrations.

Precision of eDNA concentration estimates

The precision of eDNA concentration estimates for both species depended on the amount of eDNA present in the stream (Fig. 3). Streams with the lowest mean eDNA concentrations tended to have RSE values equal to 100% of the mean. RSE values decreased rapidly as mean eDNA concentration increased. With three replicate samples, streams with a mean eDNA concentration around 0.05 ng·L^{-1} tended to have RSE values below the 20% threshold. The number of replicate samples collected per stream also influenced the precision of eDNA concentration estimates. Streams where more replicate samples were collected tended to have lower RSE values, at comparable values of mean eDNA concentration, than streams where only three replicate samples were collected.

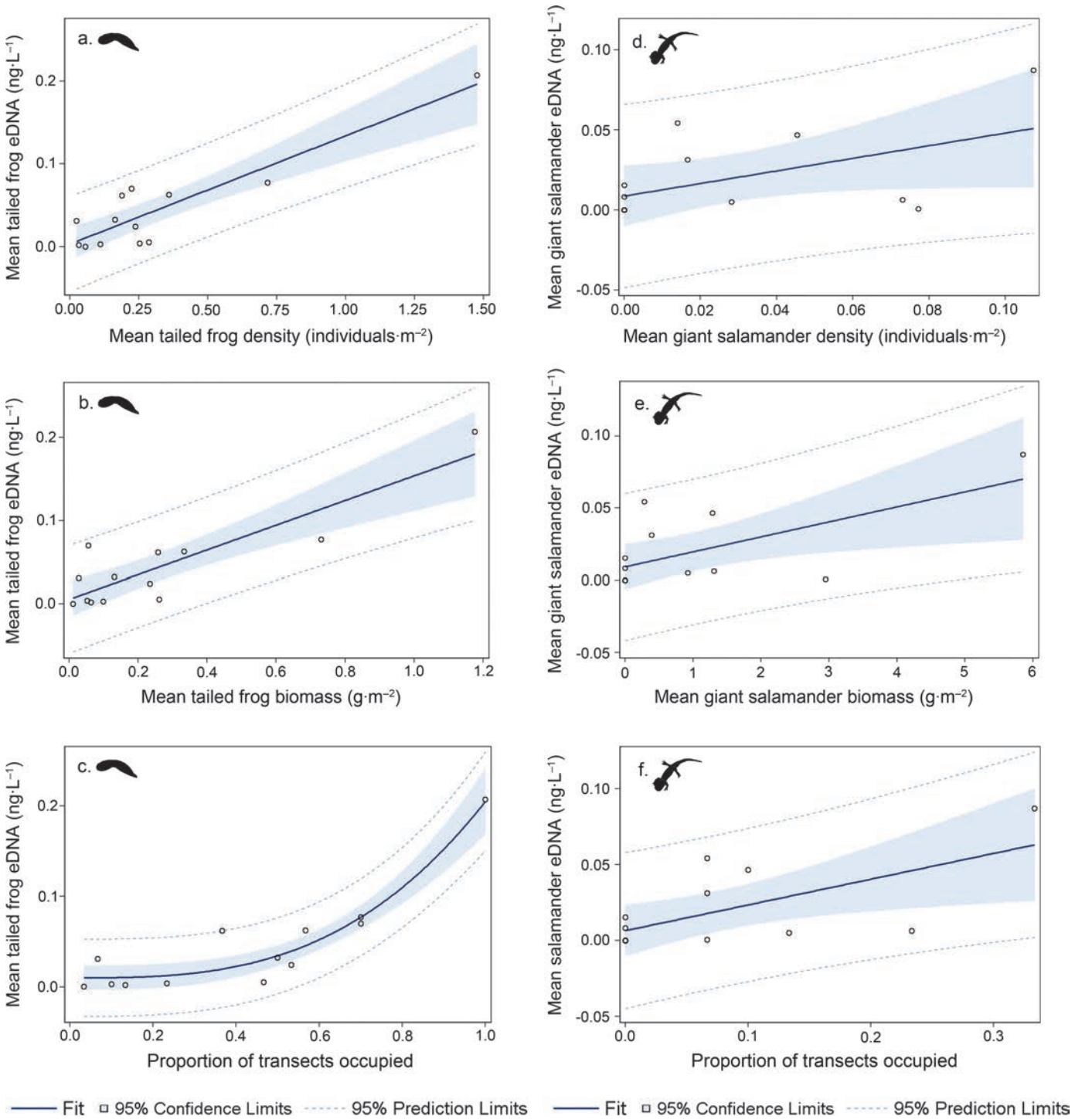
Discussion

The early success of eDNA methods for detecting freshwater species demonstrates the potential of this technique for forwarding the fields of population ecology, biogeography, and invasion biology in addition to fisheries and wildlife management. Our findings advance these potential applications in first- to third-order streams, habitats that support at-risk populations of amphibians, fishes, and other species of concern. We found that eDNA detection probabilities were not influenced by water collection methods, which allows for flexibility and convenience in the field. This finding opens the door of opportunity for cost-effective species monitoring whereby field crews can determine the distribution of a species (or assemblage of species in a biodiversity study) along a stream network simply by collecting water samples without the expense, time, training, permitting, and safety issues associated with snorkeling, electrofishing, or netting.

We found the eDNA method resulted in higher detection rates than kick-netting, especially for giant salamanders, which occurred at lower densities than tailed frogs. Salamanders were detected in two streams using eDNA, but not by kick-netting. We had detected salamanders at very low density in one of these streams in previous years, but none were captured in the year of this study. No salamanders had been captured or observed in the second stream, even after 10 years of field sampling (D.S. Pilliod and R.S. Arkle, unpublished data). This greater sensitivity of eDNA methods compared with traditional survey methods for species detection is consistent with other studies involving fish (Jerde et al. 2011) and amphibians (Dejean et al. 2012), but has not been previously assessed in high-gradient streams. By using replicate eDNA samples to estimate detection probability, eDNA methods can be used for estimating species occupancy, adjusted for imperfect detection, across a landscape (Dejean et al. 2012). However, we may find that eDNA detection rates are so high that naïve estimates of occupancy derived from eDNA methods are acceptable. For example, we found that eDNA detection rates ranged from 0.83 to 1, regardless of species or water collection method.

The amount of eDNA in our streams was related to the occupancy, density, and biomass of tailed frog tadpoles and, to a lesser extent, giant salamander larvae and paedomorphic adults. These findings, particularly those for tailed frogs, are consistent with

Fig. 1. Relationship between mean eDNA concentration and density, biomass, and occupancy (percentage of 30 transects occupied) of Rocky Mountain tailed frogs (*a*–*c*) and Idaho giant salamanders (*d*–*f*) in $n = 13$ streams. Within-stream replicates identified as outliers were removed prior to mean eDNA calculations (see Data analysis section for details and justification).



recent studies examining the density of toads and newts (Thomsen et al. 2012) and the biomass of carp (Takahara et al. 2012) in ponds. The trends for giant salamanders differed from those observed for tailed frogs probably because, across all streams sampled, giant salamander eDNA concentration was less than half that of tailed frogs (grand means = 0.02 versus 0.05 ng·L^{-1}). This finding is in accordance with the results of our RSE analyses. For salamanders, the strength of the relationship between abundance and eDNA concentration was dependent on two influential streams. One of these streams had only one salamander captured during field surveys and was very small (mean wetted width = 92 cm), which inflated the density estimate relative to the other streams. The second stream had relatively high field-estimated abundance, but inexplicably low eDNA concentrations.

We found that one challenge for high-gradient streams, which may also be problematic for other freshwater habitats, is that occasionally samples (within-stream replicates) had particularly high or low quantities of DNA resulting in statistical outliers.

Fig. 2. Salamander eDNA concentration (primary *y* axis) versus distance upstream (m) from survey start location in Weir Creek, Idaho. Each point consists of one eDNA sample. Dotted lines indicate points that are grouped into a single reach. Probability density of field-based salamander observations (secondary *y* axis; gray line), calculated using kernel-smoothed electrofishing data, versus distance upstream.

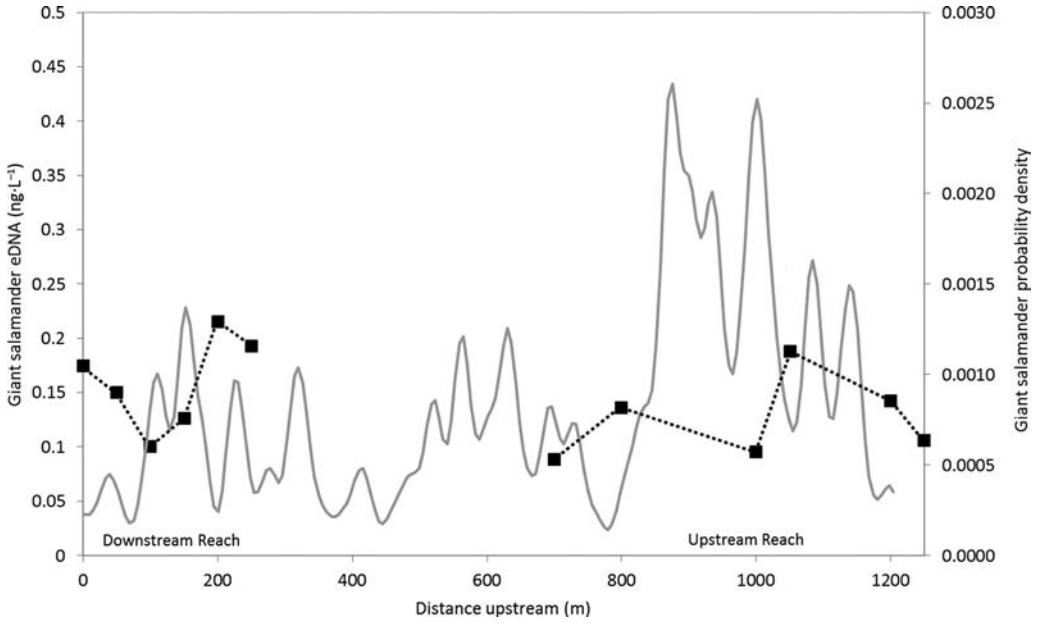


Table 3. Relationship between salamander eDNA concentrations from 11 samples collected along a 1200 m stream reach and salamander abundance, which was measured continuously in the reach using triple-pass electrofishing and removal methods.

Predictor variable	r^2	$F_{[1,9]}$	P
At eDNA sample point	0.36	5.10	0.051
Within 10 m of eDNA sample point	0.34	4.66	0.059
Within 20 m of eDNA sample point	0.25	2.93	0.121
Within 30 m of eDNA sample point	0.18	2.00	0.191
Within 40 m of eDNA sample point	0.09	0.98	0.348
Within 50 m of eDNA sample point	0.085	0.84	0.384
Within 10 m section of stream that begins 10 m upstream of eDNA sampling point	0.25	3.15	0.109
Within 20 m section of stream that begins 10 m upstream of eDNA sampling point	0.19	2.10	0.181
Within 30 m section of stream that begins 10 m upstream of eDNA sampling point	0.12	1.24	0.295
Within 40 m section of stream that begins 10 m upstream of eDNA sampling point	0.07	0.71	0.422
Within 50 m section of stream that begins 10 m upstream of eDNA sampling point	0.05	0.46	0.516

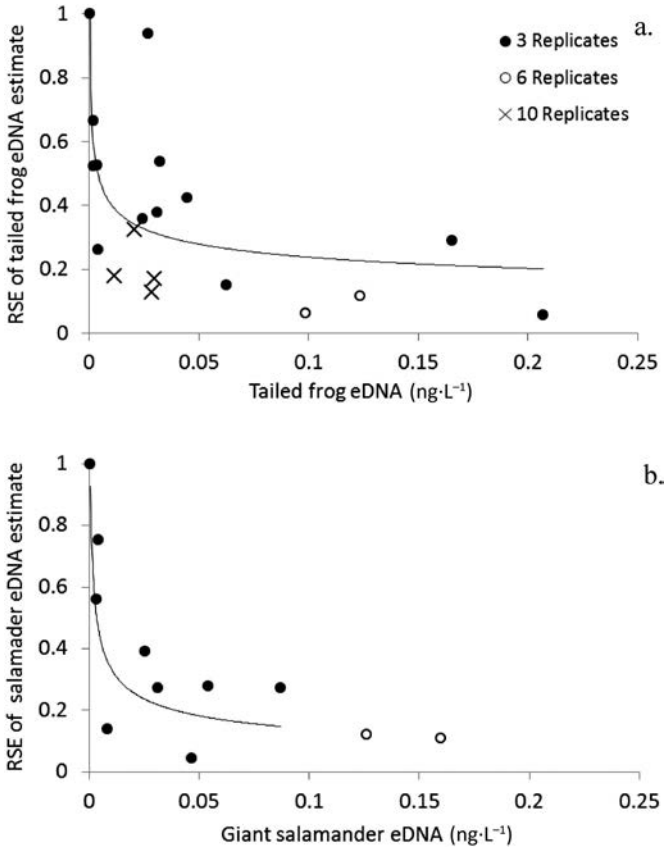
Note: See Data analysis section for description of analyses.

These outliers suggest that eDNA may move downstream as pulses of high-concentration DNA fragments or possibly whole cells. Because mitochondrial copy number varies widely among epidermal cells in amphibians, with some being mitochondria-rich, and these genetic methods are hypersensitive to low quantities of DNA, a few intact cells (especially mitochondria-rich cells) captured in a water sample may result in a spike in eDNA concentration for a given species in one replicate subsample, but not the others. While the exact cause of these spikes is unknown, it is plausible that hydrological processes in the stream or activities of animals may contribute to this phenomenon. We found that the influence of this variability on central tendency estimates was greatest in streams with the lowest eDNA concentrations and that between-replicate variability could be reduced by increasing the number of replicates or by eliminating within-stream replicate outliers in analyses. We strongly encourage the use of replicate samples to improve precision and avoid spurious conclusions that can come from sites sampled without replication. Increasing the volume of water filtered for each replicate could be an additional means of reducing error in estimates.

Despite the occasional eDNA sample with unusually high or low amounts of DNA, we found that eDNA concentrations within a stream did not vary significantly spatially (at a 50 or 450 m extent) or temporally (on a scale of 1–2 days). Contrary to our expectation, we also found that the distance a water sample was collected downstream from individual animals did not influence eDNA concentrations in the sample (at least within 0–50 m downstream). This suggests that hydrolic mixing of eDNA emissions happens quickly, and eDNA concentrations measured at a given point in a stream is representative of an upstream reach. The area of inference upstream has yet to be determined, but is likely influenced by a combination of factors such as channel morphology, flow, eDNA degradation rates, and substrate adsorption potential. We expected eDNA concentrations to increase at night because, like many amphibians, tailed frogs and giant salamanders are known to increase activity at night. However, our analysis of tailed frog eDNA concentrations over 48 h did not support this hypothesis. Collectively, these findings suggest that eDNA is moving downstream as a fairly constant "rain", but with occasional variation (as seen in subsample replicate outlier analysis).

While this study begins to fill the "striking gap" in knowledge about how field protocols influence detection of eDNA (Lodge et al. 2012), much work remains. We agree with Lodge et al. (2012) that there is a dearth of knowledge about how different environmental conditions affect the production, degradation, and detection of eDNA. Persistence and limits of detection have only begun

Fig. 3. Relative standard error (RSE) of eDNA concentration estimate versus mean eDNA concentration estimate in each stream for (*a*) Rocky Mountain tailed frogs and (*b*) Idaho giant salamanders. Each point represents a single stream. Solid lines are fitted curves only for streams where three replicate samples were taken. The RSE values were lower in streams where more replicate samples were collected. No outlying points were excluded from any calculations.



to be investigated (Dejean et al. 2011; Takahara et al. 2012). Factors such as flow rates, currents, water density and chemistry, UV-B exposure, and other conditions likely influence DNA persistence. Besides environmental factors, eDNA detection may also be influenced by the behavior of organisms (i.e., where they reside in the water column), as well as their size and volume of secretions. Some of these factors may vary among seasons (Goldberg et al. 2011) and developmental state of the individual or structure of the population. Ultimately, sampling protocols may need to be adapted for different species, different life history or developmental stages, and environmental conditions. As this process unfolds, the application of eDNA methodologies in research and management will likely grow rapidly and may transform how freshwater species are sampled, surveyed, and monitored.

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