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Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams

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Abstract

Environmental DNA (eDNA) detection has emerged as a powerful tool for monitoring aquatic organisms, but much remains unknown about the dynamics of aquatic eDNA over a range of environmental conditions. DNA concentrations in streams and rivers will depend not only on the equilibrium between DNA entering the water and DNA leaving the system through degradation, but also on downstream transport. To improve understanding of the dynamics of eDNA concentration in lotic systems, we introduced caged trout into two fishless headwater streams and took eDNA samples at evenly spaced downstream intervals. This was repeated 18 times from mid-summer through autumn, over flows ranging from approximately 1–96 L/s. We used quantitative PCR to relate DNA copy number to distance from source. We found that regardless of flow, there were detectable levels of DNA at 239.5 m. The main effect of flow on eDNA counts was in opposite directions in the two streams. At the lowest flows, eDNA counts were highest close to the source and quickly trailed off over distance. At the highest flows, DNA counts were relatively low both near and far from the source. Biomass was positively related to eDNA copy number in both streams. A combination of cell settling, turbulence and dilution effects is probably responsible for our observations. Additionally, during high leaf deposition periods, the presence of inhibitors resulted in no amplification for high copy number samples in the absence of an inhibition-releasing strategy, demonstrating the necessity to carefully consider inhibition in eDNA analysis.

Keywords: eDNA, environmental DNA, fish, lotic, qPCR, stream

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Introduction

Detection of aquatic species using environmental DNA (eDNA) techniques has the potential to revolutionize surveys of aquatic systems. Such techniques use DNA that has become separated from source organisms and suspended in the water column to detect organisms that reside in a water body (Jerde *et al.* 2011). Initial investigations suggest that eDNA techniques have greater sensitivity to detect rare species in lentic systems than most monitoring methods (Ficetola *et al.* 2008). In several studies, eDNA approaches detected species that were not collected using traditional surveys and intensive follow-up surveys often confirmed the eDNA results (Jerde *et al.* 2011; Dejean *et al.* 2012; Pilliod *et al.* 2013). As a result, there has been a recent spike in the number of studies that evaluate the use and limitations of eDNA.

To date, aquatic eDNA techniques have been used to detect a wide variety of species, including mammals such as cetaceans and mustelids (Foote *et al.* 2012; Thomsen *et al.* 2012a), amphibians (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Thomsen *et al.* 2012a), reptiles (Piaggio *et al.* 2014), insects (Thomsen *et al.* 2012a) and fish (Jerde *et al.* 2011; Thomsen *et al.* 2012a,b; Takahara *et al.* 2013). Thomsen *et al.* (2012a) used high-throughput sequencing to detect the aquatic biota in ponds, as well as terrestrial species living in close proximity to the water. Using qPCR, detection rates in ponds with known species occurrence ranged from 82% for insect species to 100% for fish species (Thomsen *et al.* 2012a).

The high sensitivity of eDNA techniques for detecting species in standing waters is becoming increasingly clear, but studies in moving waters have had varied

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results. Although detection success of weather loach (*Misgurnus fossilis*) in ponds with confirmed presence was 100%, detection rates were only 54% in a continuous 225-km^2 running water system known to contain this species (Thomsen *et al.* 2012a). These authors attributed the lower detection rate to lower fish densities and reduced exposure time between a given quantity of water and an individual organism. Other studies have successfully detected amphibian species in lotic systems even at low densities, although detection probabilities appeared to vary seasonally (Goldberg *et al.* 2011). Pilliod *et al.* (2013) reported higher detection rates for Idaho giant salamanders (*Dicamptodon aterrimus*) using eDNA over standard kick net sampling. In a companion study, however, Pilliod *et al.* (2014) detected DNA in samples taken 5 m downstream from caged salamanders, but not 50 m downstream.

Reliability of eDNA techniques in lotic systems has important management implications (Darling & Mahon 2011). The management of imperilled species, as well as control of invasive species at incipient stages of an invasion or after attempts at eradication, require methods that detect species when individuals are rare (Ando *et al.* 1998; Gu & Swihart 2004; Lodge *et al.* 2006). Use of eDNA for species detection in streams therefore requires an understanding of the dynamics of DNA in moving water. To avoid errors about species presence, it is necessary to know when a lack of detection is indicative of the absence of the target species, as opposed to the behaviour of eDNA under variable environmental conditions. For example, dilution of DNA by high flows, increasing distance from the DNA source or the presence of PCR inhibitors could reduce detectability. Many factors could alter eDNA dynamics in unexpected ways as physical, chemical and biological processes in a stream change through time. Understanding these dynamics will help managers draw more informed conclusions from eDNA data. In addition, several studies have noted a positive relationship between organism density and eDNA copy number (Takahara *et al.* 2012; Thomsen *et al.* 2012a; Pilliod *et al.* 2013), opening the possibility that these techniques may eventually be used to infer abundance. An understanding of the effects of the above environmental factors on eDNA copy number is a necessary step towards applying these techniques for abundance estimation.

In this study, we investigated the dynamics of eDNA in small streams. Our primary objective was to assess the influence and interaction of three factors: distance from a DNA source, stream discharge and fish biomass in two separate sites. Our secondary objective was to assess variation in PCR inhibition during temporally repeated sampling. To address these objectives, we designed an experiment where we introduced caged brook trout (*Salvelinus fontinalis*) into two otherwise fishless, high-gradient, headwater streams and then collected water samples at evenly spaced intervals downstream from the captive fish. We conducted nine sampling sessions in each stream from midsummer to late fall. We analysed the extracted DNA samples using quantitative PCR (qPCR) and related the amount of target DNA to distance from the caged fish for each day of sampling. We used brook trout because they are of high conservation concern in north-eastern North America (Hudy *et al.* 2008). Furthermore, they have been introduced worldwide and are the focus of eradication and control efforts elsewhere (Dunham *et al.* 2002; Rieman *et al.* 2006).

Materials and methods

Study sites

We conducted field tests in single reaches of two high-gradient, first-order streams. Streams were selected based on electrofishing and initial eDNA surveys that failed to detect the presence of any fish. Both reaches are upstream of natural waterfalls that preclude upstream movement of fish, and both systems have brook trout below these waterfalls. One reach (42°27′N, 72°41′W) was on a tributary of the Avery Brook system in Conway, Massachusetts and the other (42°23′N, 72°25′W) was on a tributary of the Amethyst Brook system in Pelham, Massachusetts. Map-based stream gradients were 50 m/km for Amethyst and 74 m/km for Avery. Both streams consist of riffles interspersed with occasional pools flowing over cobble to pebble streambeds. Mixed hardwood forest with a dense canopy surrounds both systems. Though discharges overlapped for the two streams, Amethyst tended to rise dramatically following precipitation, resulting in a much greater range of flows. The mean flow in Amethyst was higher than the highest flow in Avery. Therefore, for purposes of clarity, we hereafter refer to Avery as Small (SM) and Amethyst as Large (LG) (Figure S1, Supporting information).

At each site, a small pool was selected as a location to place a cage. The cage consisted of an aluminium frame 0.90 m long, 0.55 m wide and 0.48 m high with plastic mesh attached to the frame. Sampling sites were set up at intervals downstream from the cage by driving a section of steel rebar into the streambed. This ensured that samples were taken from the same locations over the course of the study. However, the character of these sites was subject to change with variation in flow. Because sites were evenly spaced, some sites were located in pool habitat, while others were located in riffles. The rebar markers were positioned so that they were as near to the centre of the stream as possible without having to wade into the stream to access them. The first site was 27.5 m downstream from the cage, with the remaining sites at

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26.5-m intervals downstream. This yielded a total of nine sites from 27.5 to 239.5 m downstream from the cage. We selected these distances after a pilot study suggested that eDNA copy numbers were negligible at approximately 240 m in one of these sites (SM). In SM, several small groundwater seeps entered from the side between sampling sites two and five. There were no visible inputs to the flow in LG.

Stream flow measurements

In LG, discharge was measured with a flowmeter using the midsection method (Gore 2006) while conducting eDNA sampling (see below). During low flows (<2.5 L/s), discharge was also measured with a weir and the volumetric method (Gore 2006). We placed a container of known volume below a notch in the weir and measured the time required to fill the container. Measurements were taken approximately 10 m downstream of the final sampling site about 30 min prior to the start of sample collection.

In SM, a series of flow measurements were also taken using these methods, but on separate dates from eDNA sampling. These flows were related to those at a gauging station located on a nearby river (USGS 01171500 Mill River at Northampton, MA). We ran a linear regression on these data using the gauging station flows as the independent variable and the measured SM flows as the dependent variable. We used the resulting equation ($N = 22$; $r^2 = 0.93$) to infer SM flows at the time samples were collected.

In LG, flows ranged from 1.07 to 96.00 L/s, with a mean flow of 27.70 L/s. In SM, flows ranged from 1.36 to 9.52 L/s, with a mean flow of 5.48 L/s (Table 1). Mean wetted width in SM at ten sample sites (see below) was 1.19 m at a flow of 3.22 L/s. Mean wetted width in LG at ten sample sites was 3.35 m at a flow of 31.38 L/s.

Field sampling

We collected eDNA samples by passing 6 L of stream water through a 1.5-micron glass fibre filter (GE Healthcare, Pittsburgh, PA) using a peristaltic pump (Geotech Environmental Equipment, Inc., Denver, CO). All equipment that contacted water or the filter, such as tubing, filter holders, and forceps, was sterilized with a 10-min exposure to 10% bleach prior to sampling and transported to the study site in a sterilized cooler. After water was filtered, we used sterile forceps to place filters into 15-mL conical tubes and immediately stored them on dry ice until arrival at the laboratory, where they were transferred to a −20 °C freezer until processing. In some cases, filters became clogged prior to filtering 6 L. When this happened, we placed the clogged filter into a 15-mL conical tube and used a fresh filter to filter the remaining volume of water. We then placed this filter into a separate 15-mL conical tube.

Table 1 eDNA sampling session information with session number, stream, date of sampling, total biomass of caged fish and stream flow

Session	Stream	Date	Biomass (g)	Flow (L/s)
SM-1	SM	7/11/2012	90.1	1.36
SM-2	SM	8/14/2012	95.4	2.22
SM-3	SM	9/22/2012	68.2	4.37
SM-4	SM	10/3/2012	98.8	6.66
SM-5	SM	10/17/2012	108.7	5.80
SM-6	SM	10/27/2012	76.0	7.23
SM-7	SM	11/3/2012	74.7	9.52
SM-8	SM	11/10/2012	93.1	6.23
SM-9	SM	11/17/2012	73.9	5.94
LG-1	LG	8/23/2012	115.6	1.07
LG-2	LG	8/29/2012	104.3	1.15
LG-3	LG	9/15/2012	95.4	2.29
LG-4	LG	9/29/2012	125.3	71.80
LG-5	LG	10/6/2012	119.0	12.00
LG-6	LG	10/13/2012	168.1	5.65
LG-7	LG	10/20/2012	141.6	96.00
LG-8	LG	11/7/2012	110.4	15.10
LG-9	LG	12/1/2012	114.7	9.72

Each sampling session took 2 days. On day 1, we took two samples, 1 m and about 170 m below the cage site, to use as stream negative controls. After taking these samples, we placed five brook trout into the cage. Trout were collected from nearby streams by electrofishing and the weight and length of each fish was recorded. Individual fish size ranged between 8.4 g and 38.7 g. Total biomass per session ranged from 68.2 g to 168.1 g (Table 1).

Sampling during the second day of each session began 24 h after the fish were placed into the cage. We began sampling at the downstream-most site and worked upstream, using a new filter holder for every sample. We took samples using the peristaltic pump by fastening the free end of the tubing to the rebar sample marker with a steel clip. After completing each session, we removed the fish from the cage and returned them to their source stream after clipping the anal fin to ensure that each fish was used only once.

Sample sessions were conducted from 11 July 2012 to 1 December 2012 (Table 1). A minimum of 5 days elapsed between sampling sessions to permit flushing of DNA from the study reaches. We conducted 10 sessions at each stream but had to discard one session from each. We excluded one SM session during a low-water period because flow became intermittent between the cage and downstream sites. We excluded one LG session because of observed DNA amplification in a stream negative control.

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DNA extraction and qPCR

DNA extraction and PCR set up were done in a room set aside for this purpose and kept separate from all PCR machines and post-PCR products. We performed extractions using a commercially available kit (Powerwater, MoBio Laboratories, Inc., Carlsbad, CA) designed specifically for processing water-derived DNA samples. This kit uses a silica column to capture DNA, while contaminants are washed through the column. All DNA was eluted from the column using 100 μL of sterile TE pH 8.0 (Integrated DNA Technologies, Coralville, IA) and placed at −70 °C until PCR processing. For samples that required two filters (i.e. one became clogged), the two filters were processed separately until loading upon the column, at which point they were loaded onto the same column and processed as one sample for the remainder of the protocol.

We designed a TaqMan© MGB probe targeting a region of the mitochondrial cytochrome *b* region. We used information available through GenBank to maximize probe base-pair mismatches between brook trout and closely related species (Wilcox *et al.* 2013). This probe was used as the basis for a custom assay obtained from Life Technologies (Carlsbad, CA). We used the BRK1 assay described in Wilcox *et al.* (2013). Primer sequences were F-5′ CCATGAGGGCAAATATCC TTCTGA and R-5′ TCATTGTACAAGGGCACCTCCTA, and the FAM-labelled probe sequence was 5′ CTCC TCTCTGCTGTACCC. The assay primers spanned a 90-base-pair segment of cytochrome *b*.

Initial TaqMan qPCRs were run on a Stratagene MX3005P qPCR System (Agilent Technologies, Santa Clara, CA) using 5 μL of nanopure H_2O, 10 μL of 2× TaqMan Genotyping Master Mix (Life Technologies) (hereafter 'Genotyping Master Mix'), 1 μL of 20× custom TaqMan gene expression assay containing the primers and the probe (Life Technologies) and 4 μL of extracted DNA. Upon observing evidence for PCR inhibitors, all PCRs were rerun using the same conditions with the exception that 10 μL of 2× TaqMan Environmental Master Mix 2.0 (hereafter 'Environmental Master Mix') was used in place of Genotyping Master Mix. Thermal conditions for qPCR were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

We included a standard curve consisting of the target sequence in a linearized and purified plasmid vector on each qPCR plate. The standard curve consisted of 5-fold serial dilutions of the vector in sterile TE from 156,000 copies per reaction to 10 copies per reaction, for a total of seven dilutions. We used a Qubit 2.0 Fluorometer (Invitrogen – Life Technologies, Carlsbad, CA) to measure the stock concentration of the vector prior to dilution. Immediately on preparation, dilutions were aliquoted into tubes containing a volume sufficient for one standard curve, kept at −70 °C until use and thawed and used once. Each PCR plate included samples from one session (including the negative controls), a standard curve and a triplicated no-template control (NTC) to test for contamination. Each individual sample was run in triplicate, and the mean value was used for subsequent analysis. For all qPCR reactions, standard curve R^2 values were ≥0.99 and PCR efficiencies ranged between 90.4% and 101.1%.

Test of PCR inhibition

We used 'Ct shift' between samples with the same number of known target DNA copies as a measure of the relative degree of inhibition (Flekna *et al.* 2007; Volkmann *et al.* 2007). Ct is defined as the number of cycles required for enough amplified PCR product to accumulate that it crosses a threshold recognized by the qPCR instrumentation. Ct is inversely related to starting quantity of target DNA in a reaction and is used to calculate this quantity (Heid *et al.* 1996). Presence of PCR inhibitors will shift (delay) the Ct for a given quantity of template DNA.

To test for inhibition in the DNA samples, we spiked aliquots of 18 stream negative controls (one triplicate for each session) with 250 copies of the standard curve sequence. We ran each of these on one qPCR plate using the inhibition-prone Genotyping Master Mix. We also included a triplicate sample that contained 250 copies of the standard curve sequence spiked into TE instead of stream negative control. We compared the Ct of each spiked stream negative control with the Ct for the spiked TE sample to measure inhibition-induced Ct shift for each session. To calculate inhibition-induced Ct shift, we subtracted the Ct for the TE sample from the Ct for each stream negative control. Because the maximum number of cycles was 45, if a sample had no amplification, we assigned it a Ct of 45 for this test.

After observing strong inhibition-induced Ct shifts for the Genotyping Master Mix (see 'Temporal dynamics of inhibition' in Results), we tested for inhibition with the Environmental Master Mix following the same procedure. These tests were run individually on the same plates as the corresponding sample session, resulting in an equal number of spiked TE samples and spiked stream negative controls. For the Environmental Master Mix samples, we conducted a *t*-test of the mean Ct values obtained from the spiked stream negative controls and the mean Ct values obtained from the spiked TE samples for each session and we report the mean difference between these values (inhibition-induced Ct shift).

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Statistical analysis

We conducted separate analyses for each stream using mixed models to account for correlation within the repeated measurements of each session (Pinheiro & Bates 2000; Zuur *et al.* 2009). To account for these correlations, we grouped observations by session, treating individual sampling sessions as subjects, and included random effects for the within-subject intercept and slope (by distance) (Pinheiro & Bates 2000; Zuur *et al.* 2009). We analysed streams separately because of the limited overlap in flow between sites. We used the glmmADMB package (Bolker *et al.* 2012; Fournier *et al.* 2012) in R ver 3.0.0 (R Core Team 2013). We used DNA copy number rounded to the next highest integer as the response. To model these count data, and to account for overdispersion, we used a negative binomial distribution (Zuur *et al.* 2009). We included distance, biomass and flow as model predictors. We divided distance by 100 to give the effect per 100 m and flow and biomass by 10 to give the effect per 10-unit increments. All three of these explanatory variables were *z*-score-standardized by subtracting the mean and dividing by one standard deviation (Gelman & Hill 2007). To select the final fixed effects structure of the model, we examined models with 1–3 two-way interactions or only the main effects. We then ranked the eight resulting models by AIC (Tables S1 and S2, Supporting information) and selected the model with the lowest AIC (Johnson & Omland 2004). Following model fitting, we plotted fitted values against Pearson residuals to ensure no patterns were present (Zuur *et al.* 2009).

Results

Flow, distance and biomass effects

All samples (N = 162) and nearly all individual triplicate PCRs (469 of 487) across our 18 sessions had positive detections of brook trout DNA when using the inhibition-resistant Environmental Master Mix (Fig. 1). All sessions showed measureable amplification up to 239.5 m from the cage, and there was considerable variation

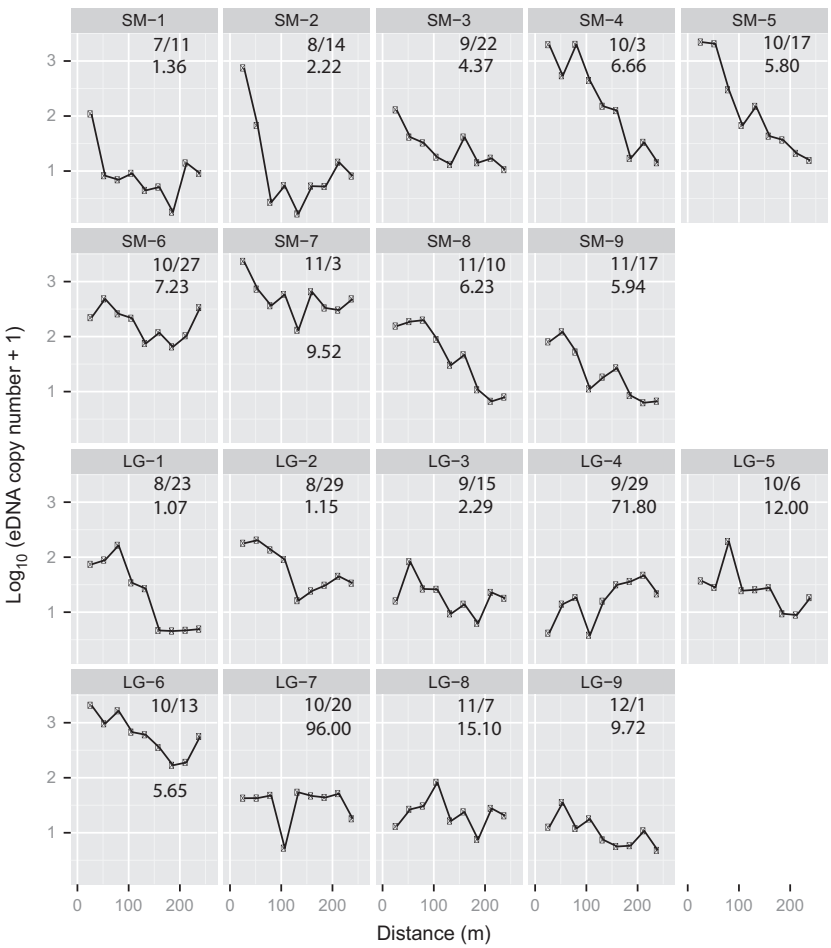

Fig. 1 Log_{10} (eDNA copy number + 1) plotted against metres downstream of caged fish for each session. Sessions in LG are shown in the bottom two rows, while sessions from SM are shown in the top two rows. Sessions are arranged chronologically within streams, starting from the upper left. Date of session is in the upper right corner of each panel and below this is flow (L/s). Note the use of the $\log_{10}$ scale on the *y*-axis. The water collection sites were located 27.5–239.5 m downstream from the cage.

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among sessions in the eDNA signal as a function of distance (Figs 1 and 2). The mean count at 239.5 m was 88 DNA copies per PCR well (SD = 176).

For LG, the highest-ranked model included all main effects as well as the distance*flow interaction (Table S1, Supporting information). The main effects of distance, flow and biomass were significant (Table 2). Distance was negatively related to eDNA copy number (Table 2). At intermediate values of all other main effects, biomass had a larger effect than flow, but opposite in direction (Table 2). The distance*flow two-way interaction was also significant, indicating that these main effects are dependent upon the levels of other main effects. For example, near the cage, increasing flows resulted in decreased eDNA copy numbers, but as distance increased, increasing flows had little effect on eDNA copy numbers. This supports the observation that as flows increase, the effect of distance was reduced so that sessions with higher flows have relatively similar eDNA copy numbers at all distances (Figs 2 and 3a).

For SM, the highest-ranked model included all main effects as well as the distance*biomass interaction (Table S2, Supporting information). The main effects of distance and flow were significant (Table 3). Distance was strongly negatively related to eDNA copy number (Fig. 2; Table 3). In contrast to LG, the effect of flow was positive in SM. The magnitude of this effect was approximately three times greater than the effect of biomass, which was also positively related to eDNA copy number (Table 3). The biomass*distance two-way interaction was also significant, indicating that these main effects are dependent upon the levels of other main effects. For example, near the cage, increasing biomass resulted in increased eDNA copy numbers, but as distance increased, this effect diminished (Fig. 3b).

Temporal dynamics of inhibition

Results reported above reflect the use of an inhibition-reducing assay (Environmental Master Mix).

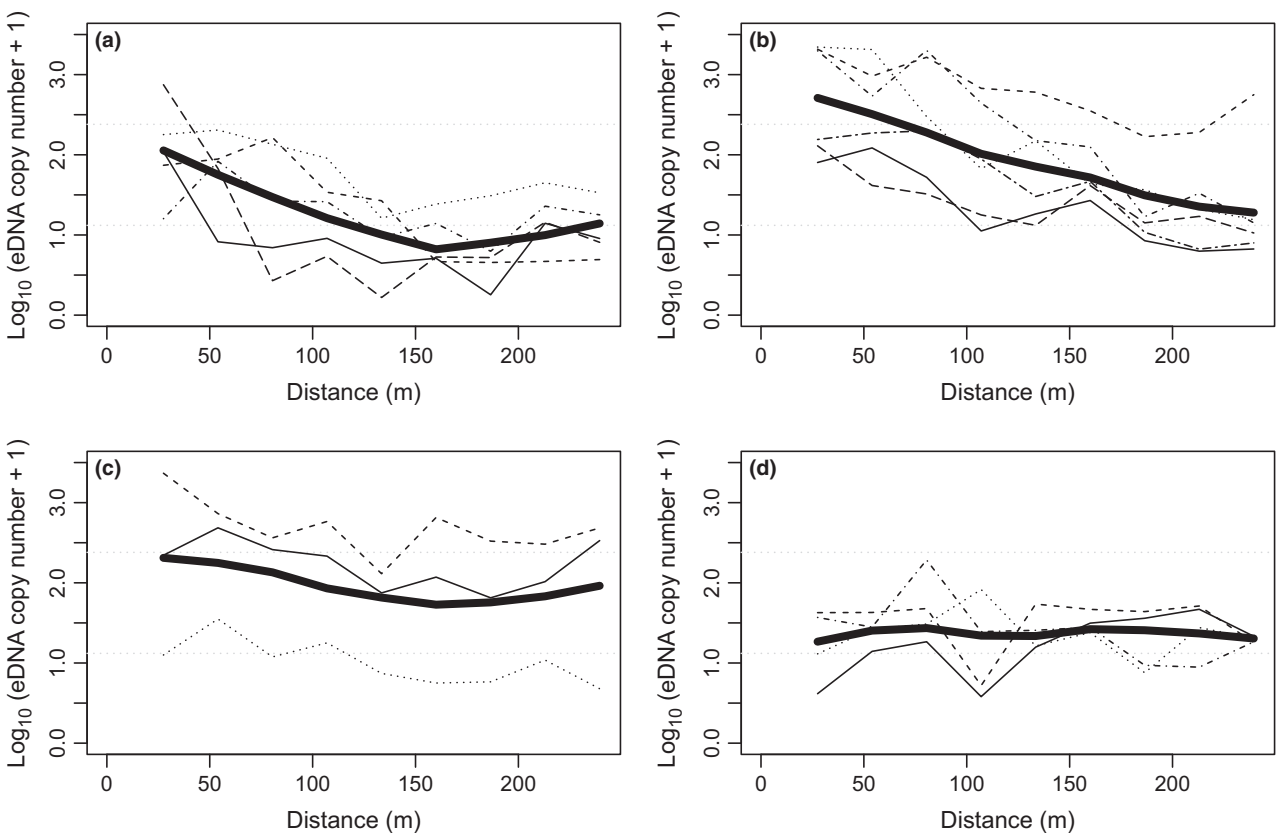

Fig. 2 Log_{10} (eDNA copy number + 1) by distance for four levels of flow. Each broken line represents an individual session (N = 18) where a session is 1 day of sampling resulting in nine evenly spaced sample locations downstream of caged brook trout. Each panel may include sessions from both SM and LG, although the highest flows (>10 L/s) were only observed in LG. The dark line is a loess smoother fit to the data within each panel. Panels are broken up into different flows as follows: a. very low flows (<4 L/s, N = 5 sessions) b. low flows (4–7 L/s, N = 6 sessions) c. medium flows (7–10 L/s, N = 3 sessions) and d. high flows (>10 L/s, N = 4 sessions).

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Table 2 Parameter estimates for the fixed and random effects of the AIC favoured model for LG. Distance was divided by 100 to give the estimate per 100 m and represents the distance from the cage. Flow was divided by 10 to give the estimate per 10-L increase in flow. Biomass was divided by 10 to give the effect per 10-g increments of biomass. These values were then z-score-standardized. Due to the z-score standardization, main effects are evaluated at the mean value of all other main effects

Random effects	Variance	SD
Intercept	0.287	0.536
Slope	0.065	0.254

Fixed effects	Estimate	95% CI lower	95% CI upper	*P*-value
Intercept	3.671	3.291	4.052	<0.001
Distance	−0.442	−0.669	−0.214	<0.001
Flow	−0.558	−0.985	−0.130	0.011
Biomass	1.063	0.638	1.488	<0.001
Dist*flow	0.383	0.142	0.625	0.002

Inhibition-induced Ct shift strongly decreased for all sessions with Environmental Master Mix (inhibition-reducing) compared with Genotyping Master Mix (noninhibition-reducing; Fig. 4). Mean Ct values for the spiked stream negative controls (M = 29.74, SD = 0.80) were not significantly different than mean Ct values of the spiked TE samples (M = 29.89, SD = 0.78) when using Environmental Master Mix ($t(34)$ = 0.58, P = 0.57). Mean Ct shift for the Environmental Master Mix runs was negative (M = −0.15, SD = 0.26) and therefore was negligible. Mean Ct shift was much greater for Genotyping Master Mix runs (M = 8.59, SD = 6.26). Some sessions that were strongly inhibited with Genotyping Master Mix had very high eDNA copy numbers when examined with Environmental Master Mix (Fig. 5). Even the least inhibited sample with Genotyping Master Mix had increased eDNA quantities with Environmental Master Mix (Fig. 5).

There was a strong seasonal component to inhibition. Without the use of the inhibition-reducing assay, eDNA became negligible to absent during autumn, coincident with an increase in leaf litter (Fig. 4; Figure S2, Supporting information). SM had one sample and LG had five samples with complete inhibition (Ct shift = 15.43). In LG, there were two additional instances of complete inhibition at other times (Figures S2 and S3, Supporting information).

Discussion

The most compelling results from these experiments are 1) that we were able to detect DNA 100% of the time, even at those locations that were furthest downstream, and 2) that inhibition has the potential to completely mask high eDNA copy numbers, a factor that has received little attention in the animal eDNA literature, but which can profoundly affect our understanding of detectability. Indeed, we designed these experiments based on preliminary sampling that was affected by

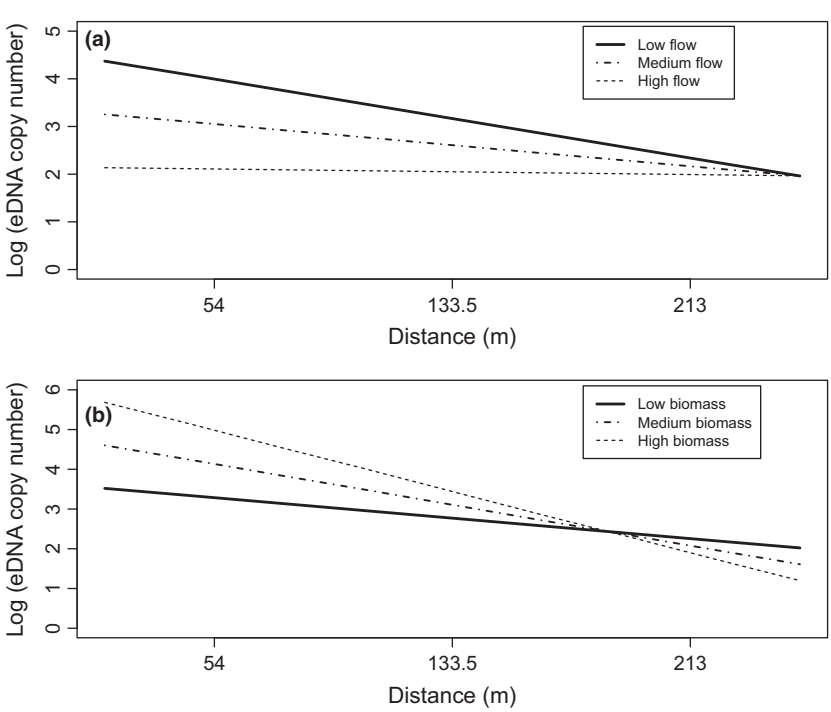

Fig. 3 Illustration of the two-way interactions in LG (a) and SM (b). Shown in (a) are the model predictions, as natural log of eDNA copy number, for three levels of flow in LG when biomass is held constant. Shown in (b) are the model predictions for three levels of biomass in SM when flow is held constant.

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Table 3 Parameter estimates for the fixed and random effects of the AIC favoured model for SM. Distance was divided by 100 to give the estimate per 100 m and represents the distance from the cage. Flow was divided by 10 to give the estimate per 10-L increase in flow. Biomass was divided by 10 to give the effect per 10-g increments of biomass. These values were then z-score-standardized. Due to the z-score standardization, main effects are evaluated at the mean value of all other main effects

Random effects	Variance	SD
Intercept	0.211	0.460
Slope	0.030	0.174

Fixed effects	Estimate	95% CI lower	95% CI upper	*P*-value
Intercept	4.300	3.948	4.649	<0.001
Distance	−1.025	−1.232	−0.818	<0.001
Flow	1.193	0.812	1.573	<0.001
Biomass	0.335	−0.046	0.717	0.085
Biomass*dist	−0.512	−0.728	−0.295	<0.001

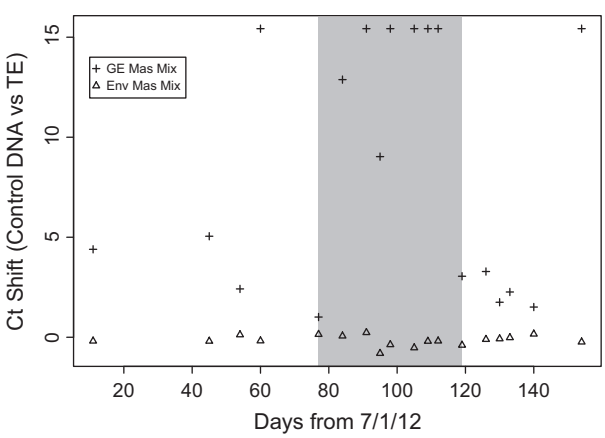

Fig. 4 Ct shift between stream negative control DNA spiked with 250 copies of the standard curve sequence and an equivalent PCR reaction with TE in place of stream negative control. GE Mas Mix refers to samples run using the initial, inhibition-prone master mix. Env Mas Mix refers to samples run using the inhibition releasing Environmental Master Mix. Grey bar represents the time period where leaf deposition was occurring, determined by observation of fresh leaves (not yet turned brown) present in the stream. Photographs taken during sampling were used to delineate this period.

inhibition; it is clear that the downstream limits of detection occur further downstream than 239.5 m. Beyond these findings, many of these results were unexpected. We expected a decline with distance, and in several of the lower-flow sessions, there were declines. However, at the lowest flows, declines levelled out and copy numbers at downstream locations were remarkably constant. Additionally, at the highest flows in LG, eDNA copy numbers were relatively consistent across samples within a session, with patterns approaching flat lines. Further, flow appeared to have relatively little effect on downstream eDNA copy numbers despite the potential for dilution.

Though we observed an effect of flow, the direction of this effect was opposite in the two streams. In LG, which had a broader range of flows, flow had a negative effect on eDNA copy number, whereas in SM flow was positively related to eDNA copy number. The general pattern of eDNA counts across the two streams consists of a large positive intercept with a steep negative slope at the lowest flows. As flows increased, the slope increased (became less negative), without a decrease in intercept. As flows peaked, the slope approached zero and the intercept declined. The two highest flows in SM approached a flat line (Fig. 1), although DNA copy numbers (intercept) remained high. We predict that higher flows in SM (outside the range of our observations) would lead to a flattened slope and lower overall eDNA copy numbers (reduced intercept), similar to patterns observed in LG. Without observations at higher flows in SM, this remains a conjecture. However, the combined results from both streams suggest that a general interaction occurs between flow and distance from source (Fig. 2).

We suggest the primary mechanisms responsible for these patterns involve cell settling and dilution. Use of a 1.5-micron filter probably sampled fine particulate organic matter (FPOM) such as partial, whole and clumped cells as the DNA source rather than molecular or 'free' DNA (Kiffney *et al.* 2000). Settling and storage of FPOM in the streambed might occur more quickly and over shorter distances at lower flows, making the cells unavailable for sample in the water column at greater distances from the source (Minshall *et al.* 2000; Wipfli *et al.* 2007). In SM, where all flows were under 10 L/s, the positive relationship between eDNA copy number and biomass was diminished at distances further from the cage. Such settling of material at these low flows could help to explain this observation. Studies indicate that the majority of FPOM transport in streams does occur during high flows (Bilby & Likens 1979; Kiffney *et al.* 2000; Wipfli *et al.* 2007) and that transport of suspended FPOM ceases once gravitational forces exceed upward, turbulent forces (Webster *et al.* 1987). At the same time, increased water volume at higher flows would serve to dilute the high DNA counts observed nearer the cage at low flows. The combination of these processes, particularly the effects of turbulent forces and dilution, may result in the observed flatter lines and low intercepts at the highest flows in LG. Additionally, Cushing *et al.* (1993) noted that FPOM might be resuspended and exported after initial deposition in the streambed. Potentially, this may occur more frequently under more turbulent, higher flows (Fisher *et al.* 1983).

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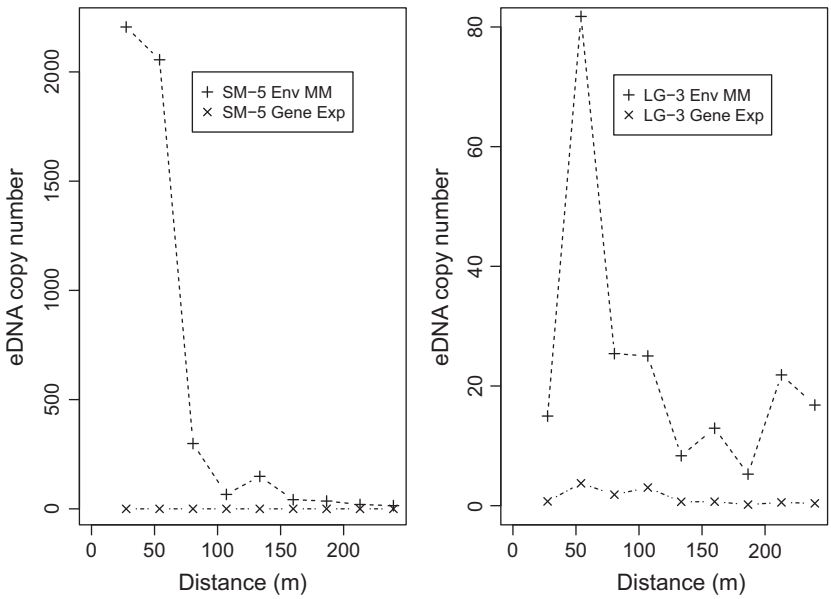

Fig. 5 Some samples that were completely inhibited on the first run had very high eDNA copy numbers after rerunning with Environmental Master Mix (left panel). Even the least inhibited sample had higher eDNA copy numbers following reruns (right panel). Gene Exp refers to the initial PCR Master Mix that was used. Env MM refers to the inhibition releasing Environmental Master Mix.

Other potential explanations are worth considering. One possible mechanism driving the observed patterns is that more DNA is produced at higher flows, particularly if fish are increasing contact with the cage. Though we cannot rule out this possibility, increased DNA shedding at higher flows seems unlikely. Under experimental conditions, Asian carp (*Hypophthalmichthys* spp.) held in higher flow tanks did not shed more than fish in slower flow tanks (Katy Klymus, USGS Columbia Environmental Research Center, personal communication). Alternatively, at low flows, PCR inhibition may increase with distance from the eDNA source. However, the use of Environmental Master Mix eliminated most, if not all, inhibition from our samples. Another explanation for the decline in eDNA copy numbers with distance from source at low flows could be increased contributions of groundwater or riparian seeps. At low flows, dilution from groundwater seeps as DNA travels downstream from the cage could create the pattern of rapid decline with distance from cage. At higher flows, seeps would have less effect on eDNA copy number because they would be a minor proportion of the total water in the stream. Under these conditions, the expected pattern would then be lower maximum DNA counts and more consistent counts across samples within a session, which is consistent with the observed pattern. Further, we observed seeps in SM, where this pattern was stronger, but not in LG, although they could occur within the stream channel. Finally, there could be a certain amount of stream specificity of eDNA dynamics due to differences in shear stress, bed roughness and the area of transient storage zones available within the stream (Webster *et al.* 1987; Eckman 1990; Cushing *et al.* 1993; Minshall *et al.* 2000). Such characteristics could result in stream-dependent variation in eDNA dynamics.

It is clear that eDNA detection represents a powerful new tool for determining species presence in headwater streams. However, the utility of eDNA for estimating abundance or biomass in headwater streams remains uncertain. We observed a positive relationship between biomass and eDNA copy numbers in this study, which is consistent with other studies (Takahara *et al.* 2012; Thomsen *et al.* 2012a; Pilliod *et al.* 2013). However, the observed interactions between distance and flow may be confounding factors in attempts to infer abundance based on eDNA sampling in moving water. These patterns, combined with uncertainty surrounding distance from source in field settings, will complicate biomass estimation, at least at low flows. Manipulative experiments that attempt to predict biomass from eDNA copy numbers based upon distance from source and flow would be a helpful next step to determine the feasibility of using eDNA for these purposes in lotic systems.

Pilliod *et al.* (2014) noted that eDNA was undetectable almost immediately upon removal of organisms (i.e. 1 h after a target species was removed from a stream). Our results were similar. In one case, we aborted a sample after the cage became isolated from the downstream sample sites because the stream became intermittent. These remaining sites were completely cut off from the cage (i.e. DNA source) at some point during the intervening 24 h. We processed samples from the remaining downstream sites (#2–#9) and found DNA in only two. Both of these had only trace amounts of DNA (triplicate mean of 1 copy or less), showing that most DNA was flushed out of the study reach within 24 h of removal of

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the source. Studies have found that it takes eDNA several days to degrade to undetectable levels (Dejean *et al.* 2011; Pilliod *et al.* 2014), so the bulk of this was likely due to downstream transport or storage of DNA in the stream bed.

Rarely will organisms be present in one localized cluster within a stream, as was the case in our experiment. Localized spatial clustering may occur at very early stages of colonization or invasion, but organisms are likely to be more evenly distributed in established populations. We found that eDNA travels a long distance from the source, so it may be that in established populations, eDNA concentrations are dictated by an equilibrium between DNA originating from upstream sources and DNA generated from localized sources. Such a dynamic may mask the patterns we observed with distance. Within the same stream, Pilliod *et al.* (2013) found no relationship between eDNA concentrations and the densities of Idaho giant salamanders (*Dicamptodon aterrimus*) in the 50-m reach upstream. They also found no significant difference in eDNA concentrations between samples separated by 450 m within the same stream. This may be the result of equilibrium processes or possibly flow was strong enough that signals had flattened out.

Our results show that eDNA studies need to carefully consider PCR inhibition to avoid detection errors. Though all sessions had at least some inhibition, inhibition appeared to be more pronounced during autumn when there were large quantities of leaf matter in the streams. Plant matter is known to contain compounds that inhibit PCR (Demeke & Adams 1992; John 1992; Wilson 1997), so it is not surprising that leaf matter might result in increased inhibition. Additionally, LG had more samples with complete inhibition than SM, and LG had the only samples outside of peak leaf fall that had complete inhibition. The water in LG had a visible yellow to orange hue, while SM was nearly colourless. This suggests that LG may have a relatively higher concentration of plant compounds that may help to explain overall higher inhibition.

We found that inhibition could render undetectable high eDNA levels in excess of 2,000 copies. Potentially, dilution of extracted DNA is one method that can reduce inhibition during the PCR process (Abu Al-Soud *et al.* 2000; Volkmann *et al.* 2004). However, dilution can also result in a negative PCR result when copy numbers are low yet detectable (Juen & Traugott 2006). PCR facilitators such as BSA can reduce inhibition, but tend to target specific classes of inhibitors (Strand *et al.* 2011). TaqMan Environmental Master Mix has been found to do a better job of releasing inhibition over a wide range of environmental conditions, removing close to 100% of inhibition from water samples in some cases (Strand *et al.* 2011; Albers *et al.* 2013). This is consistent with the release of inhibition in our samples observed using Environmental Master Mix.

We conducted a test for inhibition by spiking samples with known copy numbers of an external DNA sequence. An alternative to this post hoc approach is to include an internal positive control within each PCR reaction (Hartman *et al.* 2005). The advantage of this approach is that each individual PCR reaction can be monitored independently for inhibition in one step. Disadvantages of this approach include the increased complexity of assay design, which requires increased optimization work (Courtney *et al.* 1999; Hoorfar *et al.* 2004). For multiplex reactions, it is also possible that internal positive controls may inhibit the amplification of the gene target as a result of resource competition (Volkmann *et al.* 2007). Most studies using eDNA to detect aquatic animal species to date have not used internal positive controls (but see Goldberg *et al.* 2011, 2013; Pilliod *et al.* 2013, 2014).

Conclusion

Our results are consistent with other studies that indicate eDNA detection is a powerful tool for assessing the presence of organisms in high-gradient streams (Pilliod *et al.* 2013). We successfully detected brook trout at relatively low biomass (0.07 to 0.17 kg) in all samples and all distances, regardless of flow. This high rate of detection occurred even after only 24 h following introduction of caged fish into the streams. That we detected DNA at 239.5 m in all sessions suggests that DNA may travel much further downstream, possibly on the order of kilometres. The eDNA of lake-dwelling invertebrates has been detected several kilometres downstream from the source population in a lake outlet, supporting this conclusion (Deiner & Altermatt 2014). It remains to be seen whether eDNA techniques will be reliable methods for determining organism abundance in high-gradient streams. We observed a positive relationship between biomass and eDNA copy numbers. However, significant interactions between distance, flow, and biomass, as well as possible stream-specific effects, indicate that models estimating abundance from eDNA concentrations will need to account for these effects.

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Data Accessibility

Raw data collected from the study sites, including qPCR copy number per PCR reaction for each 6 L water sample at each measured distance are available at DRYAD: doi:10.5061/dryad.7j3g5. This data includes biomass per session, mean raw DNA copy number per PCR reaction per sample, flow per session, and session dates. Ct shifts for spiked negative stream controls can be found in Fig. 4.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Photographs of the two study sites. The top two photos are from the same location in LG during low flow (left) and high flow (right). The bottom two photos are from the same location in SM during low flow (left) and high flow (right). Note that the LG photos were taken at a weir used for measuring flow and are about 10 m below the study reach.

Fig. S2 Results using the inhibition-prone Genotyping Master Mix. Log_{10} transformed (eDNA copy number + 1) by distance for each sample. The date, as month/day, for each sample is in the upper right corner of each panel. Flow in L/s appears below the date. The lower two rows are LG samples and the upper two rows are SM samples. Within a stream, panels appear in the order of sampling date, from upper left to lower right. The Large X sample was not included in further analysis because there was amplification in one of the negative controls when run using Environmental Master Mix.

Fig. S3 Inhibition-induced Ct shift plotted against sessions in chronological order on the *x*-axis. Inhibition-induced Ct shift is a measure of the delay in Ct as a result of inhibition and is a relative measure of inhibition. Greater shifts in Ct indicate stronger inhibition. The largest Ct shift (15.43) indicates complete inhibition that resulted in no amplification of DNA.

Table S1 The lower table shows the structure of the competing fixed effects models for LG. Dis = Distance, Fl = Flow, Bi = Biomass. An X below the variable in the column indicates that it was included in the model. The upper table shows the AIC and ΔAIC for each model with models listed from lowest to highest AIC.

Table S2 The lower table shows the structure of the competing fixed effects models for SM. Dis = Distance, Fl = Flow, Bi = Biomass. An X below the variable in the column indicates that it was included in the model. The upper table shows the AIC and ΔAIC for each model with models listed from lowest to highest AIC.

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