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Modelling the transport of environmental DNA through a porous substrate using continuous flow-through column experiments

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Detecting environmental DNA (eDNA) in water samples is a powerful tool in determining the presence of rare aquatic species. However, many open questions remain as to how biological and physical conditions in flowing waters influence eDNA. Motivated by what one might find in a stream/river benthos we conducted experiments in continuous flow columns packed with porous substrates to explore eDNA transport and ask whether substrate type and the presence of colonized biofilms plays an important role for eDNA retention. To interpret our data, and for modelling purposes, we began with the assumption that eDNA could be treated as a classical tracer. Comparing our experimental data with traditional transport models, we found that eDNA behaves anomalously, displaying characteristics of a heterogeneous, polydisperse substance with particle-like behaviour that can be filtered by the substrate. Columns were quickly flushed of suspended eDNA particles while a significant amount of particles never made it through and were retained in the column, as calculated from a mass balance. Suspended eDNA was exported through the column, regardless of biofilm colonization. Our results indicate that the variable particle size of eDNA results in stochastic retention, release and transport, which may influence the interpretation eDNA detection in biological systems.

1. Introduction

Environmental DNA (eDNA) techniques that identify the presence of genetic material (e.g. free DNA, cells, tissue, faeces) of a target species in water samples are a promising and emerging approach for the detection of rare and invasive species in aquatic systems [1,2]. Sampling for target species using eDNA is beneficial because it is non-intrusive and enables detection without direct observation [3], allowing for detections at lower abundance compared with conventional sampling methods [4]. The application of eDNA detection techniques is diverse and include detecting threatened and endangered species where eDNA sampling does not cause harm through capture [5]; early detection of invasive species for purposes of eradication or spread prevention [1,4,6] and estimation of population biomass, abundance or composition of aquatic communities [7–10].

While recent studies have demonstrated that eDNA is a powerful tool for detecting target species in aquatic ecosystems [11], the majority of studies to date focus on detection from water in mesocosms [2,12–16] or experimental/natural ponds [1,7–9,11,12,16]. A few studies have used eDNA survey methods in stream or river water samples for detection of amphibians [9,15,17], molluscs [18], fish [6,9,13,19] and invertebrates [20]. The spatial distribution of eDNA, however, has only recently been explored, demonstrating that eDNA in rivers

can be transported potentially over large distances [20–23]. Importantly, this quality may complicate interpreting what a detection actually means relative to where a species might be present.

Streams and rivers are naturally complex systems [24]. In particular, while there may be a fast flow in the main channel, water is actively and continuously exchanged with the surrounding porous substrates (i.e. a process called hyporheic exchange) and flow velocities in these porous media are typically orders of magnitude slower than in the open flow channel. Therefore, in flowing waters, the target organism may be some distance away from where the eDNA in water is collected [19], and additionally, eDNA fate may be impacted by the hydrologic complexity of streambeds. Surface–subsurface exchange may trap eDNA in sediment interstices [25], on biofilms [26,27], or organic matter (OM) may bind eDNA [28]. Furthermore, retention of eDNA particles in streambeds could lead to positive detection of eDNA owing to a delayed release back into the open water, even when source species are no longer present. While the heterogeneity of the flow system already adds a great deal of complexity even for the transport of a simple conservative species, recent research suggests an additional complexity in the case of eDNA. More specifically, eDNA is not a simple monodisperse phase as it can consist of a range of particle sizes and materials that can include free DNA, cells, tissue fragments [1,2]; thus, eDNA is polydisperse (e.g. 0.2–180 µm for carp [29] and brook trout [30] eDNA). Understanding and ultimately predicting the fate and transport of eDNA in natural flows is an inherently challenging problem with multiple layers of complexity that need to be tackled individually to ultimately build a clear and comprehensive modelling framework. Despite pressing scientific and societal needs, our current understanding of eDNA transport and persistence in porous substrates, such as complex stream and river beds, remains imprecise and limits our ability to adequately interpret eDNA detection in complex systems [31,32]. To date, studies of eDNA through porous media are limited if not non-existent and, therefore, in this work we focus on that aspect of the larger problem.

To address knowledge gaps associated with eDNA detection in flowing water, we focused on the slow flow-through porous media and conducted a series of continuous flow column experiments. As a first-order attempt, we interpret the experimental results using a traditional one-dimensional advection–dispersion–reaction transport model. We estimated transport parameters of eDNA through these experimental columns as if DNA behaved as a typical monodisperse particle or solute and compared resulting models with empirical data. While this model is simple and will never capture the full complexity inherent to this system, it is to date the most common model for transport of any substance through porous media and most hydrologic environments. This is due to its parsimonious nature and ability to capture certain critical features, such as mean arrival times/concentrations, reasonable measures of spread (i.e. dispersion) around this and characteristic rates of degradation, all of which provide critical knowledge to understanding how a substance moves in a hydrologic system.

Before conducting our experiments, we chose to focus on two key questions: (i) Will eDNA be retained more quickly in a finer compared with a coarser substrate, where pore size influenced filtration mechanisms will be stronger? and (ii) Will the presence of a natural biofilm on the porous substrate influence transport of eDNA? In the case of (i), we anticipated that finer substrates would retain more eDNA and not allow it to pass as freely through the porous medium. We used substrates that might be found in a low-gradient headwater stream, such as sand ($D_{50} = 1$ mm) and pea gravel ($D_{50} = 1$ cm), which are both characteristic of the hyporheic zone of a natural system. For (ii), we anticipated that biofilm-colonized columns would homogenize the differences between substrate size, and that biofilm-colonized substrates would retain more eDNA relative to the biofilm-free columns.

2. Methods

2.1. Column experiments

We injected a solution containing eDNA from bluegill sunfish (*Lepomis macrochirus*) into flow-through columns packed with substrates of contrasting size, before and after biofilm colonization, to determine how substrate size and biology might impact the retention of eDNA in porous substrates. In all cases, we wet-packed substrate into chromatography columns of dimension 4.8 (inner diameter) × 60 (length) cm glass (Chromaflex) with Teflon fittings and attached them to a peristaltic pump via Masterflex L/S tubing [16,24]. We ran the experiments using four different porous media: clean quartz sand (hereafter S, $D_{50} = 1$ mm), clean pea gravel (PG, $D_{50} = 1$ cm), sand colonized with biofilm (SB) and pea gravel colonized with biofilm (PGB). Our experimental design is depicted schematically in figure 1 [33]. We chose PG and S substrates as they are representative of the small benthic substrate typical of a low-gradient streams. Prior to each substrate packing, we sterilized the glass column, tubing and fittings with a 10% bleach solution for 10 min to avoid possible eDNA contamination [34]. We rinsed the column and materials vigorously before each experiment to remove any residual bleach. For substrate with biofilms (PGB, SB), we incubated them in low-nutrient experimental streams and full sunlight for three weeks at the Notre Dame Linked Ecosystem Experimental Facility in South Bend, IN, USA (http://research.nd.edu/core-facilities/nd-leef/).

For each individual experiment, we pumped at least four pore volumes (PVs) of deionized water through the wet-packed column at 150 ml min^{-1} with a peristaltic pump and then transferred the inflow tube to a sodium chloride solution (concentration = 25 mg NaCl l^{-1}). We measured solute breakthrough curves (BTCs) using a YSI 3200 conductivity meter and probe cell to understand conservative transport through each column. We used chloride as a conservative tracer, which is commonly used in similar experiments; on short time scales, the low concentration of chloride used should not have been a concern (Nerenberg R 2013 (Department of Civil and Environmental Engineering & Earth Sciences, University of Notre Dame). Oral communication 2013 October). Additionally, additions of NaCl are used as conservative tracers for nutrient transport studies with no significant influence on biofilms; chloride is a biologically essential solute that typically exists in streams in concentrations that exceed biological need, and is not stressful to biofilms [35]. Additionally, in some very early experiments in our eDNA studies, we also took measurements in cases where NaCl was absent with no notable influence (CL Jerde, BP Olds, AJ Shogren, EA Andruszkiewicz, AR Mahon, D Bolster, JL Tank 2013, unpublished data).

After characterization of flow-through columns using the conservative tracer, we conducted experiments with an eDNA solution, which came from established tanks holding a steady population of bluegill sunfish. We allowed eDNA to accumulate by turning off the flow-through filtration on the tanks for 12 h prior to influent solution collection; we expected the shedding rates from bluegill fry to remain similar over time [36]. To

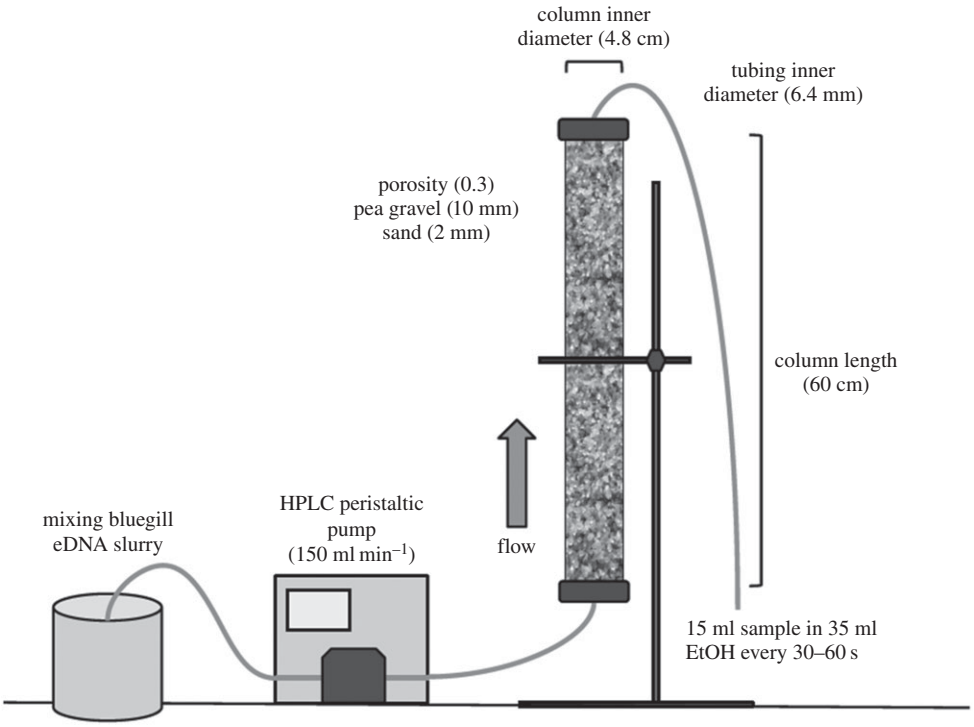

Figure 1. Schematic of the experimental set-up for column studies under saturated conditions (modified from Anders & Crysikopoulos [33]).

remove any large particles (e.g. tissue, scales, etc.) in the influent solution, we filtered the influent solution through clean 0.5 mm mesh. To keep the collected solution mixed and avoid settling and segregation, an aquarium pump continuously mixed the solution containing eDNA throughout all column experiments (pump speed = 0.15 ml s^{-1}). We followed a timing schedule determined using the conservative tracer additions. For the PG experiments, we pumped eDNA solution through each individual column (150 ml min^{-1}) and sampled every 30 s for the first 10 min, at 1 min intervals for 15 min and at 5 min intervals for 35 min thereafter. For the sand experiments, we sampled every minute for 40 min, then at 5 min intervals for 20 min. We designated time zero ($t = 0$) at the start of when we began to pump in the eDNA solution, and at $t = 18$ (PG, PGB) or 24 (S, SB) minutes we placed the tube back into fresh water to flush the column of suspended eDNA, but continued to monitor outflow to capture eDNA retention for 42 and 36 additional minutes, respectively. At 10 min intervals throughout each experiment, we took 15 ml samples directly from the influent solution ($n = 6$) using a sterilized 30 ml syringe, treating them the same as the effluent samples (see below). We performed each experiment twice with each substrate treatment, yielding two replicate runs for each substrate, for a total of eight separate experiments.

We collected all effluent samples ($n = 50$ per experiment) as 15 ml in 60 ml centrifuge Falcon tubes with 33.5 ml aliquot of 100% ethanol and 1.5 ml of a 3 M sodium acetate solution. We determined the pump flow rate of 150 ml min^{-1} using Stokes settling velocity for a 200 µm particle and estimated that 150 ml min^{-1} yielded a sufficient velocity to suspend eDNA particles (less than 200 µm). Following collection, we stored samples at −20°C until centrifugation, following the methods of Ficetola *et al*. [1] and Thomsen *et al*. [9].

After each column experiment, we collected four approximately 20 ml substrate samples directly from the column in 160 ml specimen cups for estimation of chlorophyll a (chl a—represents a relative quantification of autotrophic portion of biofilm) and OM using ash-free dry mass (AFDM—represents the total organic mass in attached biofilm) of attached biofilm. For chl a, we extracted chl from each substrate sample and measured fluorometrically using standard methods [37], expressing chl a per unit surface area of substrate. From the remaining two samples, we estimated OM by placing the approximately 20 ml of sampled substrate into 100 ml of water and mixing vigorously to loosen biofilm from the substrate; the water plus biofilm slurry was filtered onto a pre-ashed and weighted glass fibre filters (GF/F, Whatman) filtered and dried for 48 h at 60°C to measure dry mass. The filters were then ashed at 550°C for 1 h, re-wet and dried for 48 h at 60°C to measure ash-free dry mass. There was no statistical difference between chl a (*t*-test, $t = -0.41$, d.f. = 5.9, $p = 0.69$) or AFDM (*t*-test, $t = -0.46$, d.f. = 3.7, $p = 0.67$) between biofilm-colonized substrate treatments (PGB versus SB). Mean chl a was 5.32 mg cm^{-2}, and mean AFDM was 0.464 g cm^{-2}. We took biofilm and OM samples after experiments to more accurately estimate the biofilm that remained in the column over the course of the experiment, though we recognize that biological activity of the natural biofilm may have been inhibited after exposure to deionized water.

2.2. DNA extraction

For each sample, we quantified DNA concentration in solution using a standard chloroform–isoamyl alcohol extraction and quantitative PCR (qPCR) *sensu* Ficetola *et al*. [1], which allowed us to extract DNA from a small sample volume. We used a standard cetyl trimethyl ammonium bromide (CTAB) extraction method that has been used in numerous eDNA studies [1,28,36] and verified by Renshaw *et al*. [38]. Therefore, capture efficiency should not have been a concern. We centrifuged sample tubes at 15 000 RCF at 6°C for 35 min to form a pellet and poured off the supernatant, leaving pellets to dry for 5 min. We then pipetted 700 µl of CTAB into each centrifuge tube and incubated for 10 min at 60°C. After incubation, we transferred all liquid into a 2 ml microcentrifuge tube, shaking at low speed vertically for 5 min. We centrifuged each tube for 15 min at 15 000 RFM, and then transferred 500 µl of the top supernatant to a 1.5 ml microcentrifuge tube. We precipitated DNA with 500 µl cold isopropanol and 250 µl of 5 M NaCl and incubated for 1 h at 20°C. After this incubation period, we centrifuged for 15 min at 15 000 RFM and poured off the supernatant. We rinsed the

pellet twice with 150 µl 70% ethanol followed by a 5 min centrifugation. We resuspended the DNA pellet with 100 µl TE buffer (10 mM Tris, 0.1 mM EDTA) and stored at 4°C prior to qPCR. We performed each qPCR analysis within one month of the extraction date.

2.3. DNA quantification

We assayed all DNA extractions with qPCR TaqMan® primers and probe in the following 20 µl mixes: TaqMan® Environmental Master Mix 2.0 (Life Technologies), forward and reverse primers (900 nM well concentration), TaqMan® probe (125 nM well concentration), extracted DNA and sterile water. We used the following cycling parameters: a single step at 50°C for 2 min, a single step at 95°C for 10 min, and 55 cycles at 95°C for 15 s followed by 60°C for 1 min. To quantify the DNA copy number in each DNA extract, we included a synthetic DNA standard on each qPCR plate along with the DNA extracts. For the quantification standard, we synthesized a double-stranded DNA fragment by Integrated DNA Technologies based on the sequence from GenBank accession number JN389795 starting at location 14 298 and ending at location 14 797. The 500 bp fragment included the 100 bp region of the bluegill cytochrome *b* gene targeted by the assay [7] with an additional 200 bp on either side. We determined the copy number of the synthesized standard by dividing the molecular weight by Avogadro's number. We ran a serial dilution of this standard on each qPCR plate and provided a regression line from which the unknown copy numbers of the DNA extracts could be estimated. We ran all qPCR assays on a Mastercycler ep realplex real-time PCR system (Eppendorf) and analysed with Realplex v. 2.2 software. We ran each qPCR sample in triplicate to assess repeatability; if only one or two amplified, these samples were assigned 0 concentration for later analysis (as in [7]). While we did not directly test for qPCR inhibition using an internal positive standard, we ran all samples using a commercially available Environmental MasterMix, which has been found to significantly reduce the effects of inhibition on environmental samples [21]. Additionally, we did not find any indication of qPCR inhibition. All extraction negative controls and qPCR NTCs tested negative for bluegill eDNA (no Cq). The standard curve efficiency ranged from 95% to 98%, with an R^2 from 0.97 to 1.00. Based on standard curve amplification, the 95% limit of detection was 25 copies per reaction, and the lowest concentration standard (five copies per reaction) amplified 70% of the time.

2.4. Model description

As a first-order model to interpret transport from our experiments, we estimated the transport parameters of eDNA through the columns using a one-dimensional advection–dispersion–reaction transport model. For the case of the conservative solute, we use a one-dimensional advection–dispersion equation

$$R_f \frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} - v_w \frac{\delta C}{\delta x} \tag{2.1}$$

with boundary conditions

$$C(x = 0, t) = C_0(t)$$

$$\frac{dc}{dx}(x, t = 0) = 0$$

and $0 < x < 60$,

where C represents concentration, $v_w$pore water velocity, D dispersion (which accounts for both molecular diffusion and mechanical dispersion), and R represents retardation (which captures instantaneous adsorption/desorption processes that can slow transport through the column relative to pore water flow). $C_0(t)$ represents the concentration at the inlet of the tube, which is zero when pure water is introduced and the concentration of the NaCl solution when it is pumped through. We estimated transport parameters for this ADE equation (equation (2.1)), using an openly available R package based on finite difference solution (ReacTran, [39]). The input solution is supplied with a pore water volume flow rate v_w (150 ml min^{-1}) with tracer solution of concentration C (25 mg ml^{-1}) introduced at the influx boundary, $x = 0$.

For interpretation of eDNA data, we used an analogous ADE model, but with an additional first-order reaction term, which represents a first-order degradation and/or a first-order irreversible adsorption to the porous medium, such that

$$R_f \frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} - v_w \frac{\delta C}{\delta x} - k_c C. \tag{2.2}$$

For validation of the finite-difference code, we also solved the problem with an analytical Greens function-based method, yielding identical results. We used R package FME [40] to estimate parameters by fitting the simulated BTCs to the available data, with a goodness-of-fit determined by residual sum of squares.

We assumed that the eDNA solution was completely mixed in solution, and that eDNA degradation during this short experimental period (less than 3 h) was negligible [41]; as such, the reaction term predominantly represents filtration by adsorption alone. We estimated the mass balance of the equation by comparing the outflow concentrations over time with the mean influent solution concentration.

3. Results

3.1. Breakthrough curves

BTCs for the conservative NaCl solute and eDNA concentrations for all four experimental configurations are shown in figure 2. In all cases, the eDNA concentrations were normalized to 1 using the mean measured concentration of the influent solution; the chloride concentrations were similarly normalized to 1 using the plateau concentration. The rising limb for the eDNA solutions reached a 'quasi-plateau' stage within approximately 5–6 min in all experimental runs, compared with approximately 3 min for the conservative tracer. We use the term-quasi-plateau, because while for the conservative tracer a constant concentration is attained, for all eDNA cases the concentration varies erratically about some mean value (see Discussion). We also monitored the falling limb for eDNA flushing from the columns; that is, once we removed, the source of the eDNA water and pumped eDNA-free water into the column for an additional 42 (PG, PGB) and 36 (S, SB) minutes, respectively. In the non-biofilm treatments (PG and S), there was no detectable eDNA remaining in the column (grey-shaded area, figure 2*a*,*c*) after a comparable 5–6 min. However, in the biofilm-colonized treatments (PGB and SB), we did observe detectable, albeit very low concentrations of eDNA in some outflow samples for 12 (PGB) and 18 (SB) minutes after the column began flushing (grey-shaded area, figure 2*b*,*d*).

3.2. Retention

To compare retention between biofilm-colonized and biofilm-free substrate experiments, we used a mass balance approach (i.e. PG versus PGB and S versus SB), where we estimated the amount of eDNA retained relative to what

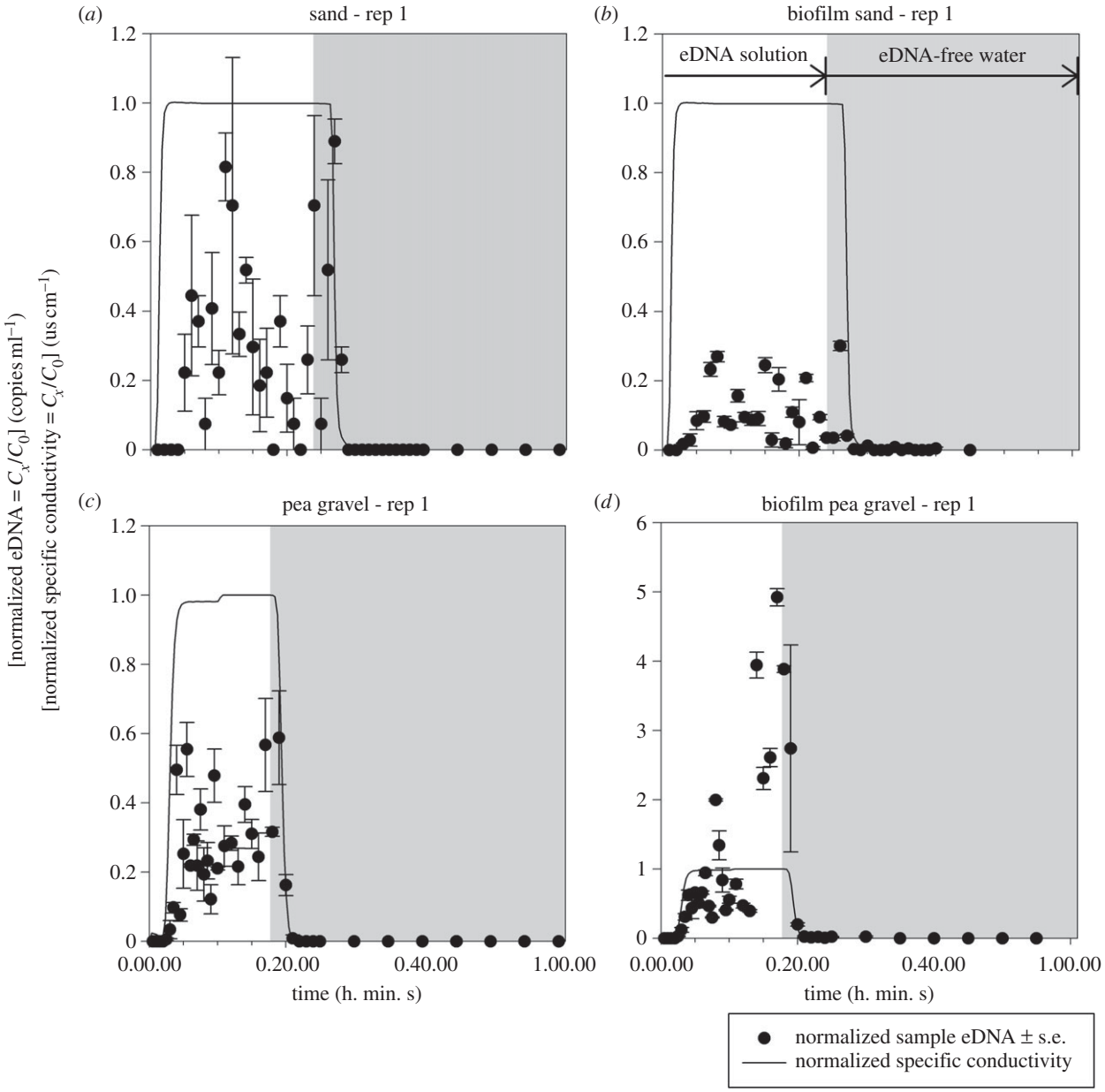

Figure 2. Normalized break through curves (BTCs) of eDNA and NaCl solutions through the column for representative column runs. Because eDNA concentrations varied among experiments, we normalized both NaCl (as specific conductivity, black line) using maximum plateau concentration, and eDNA concentrations (black points) to mean estimates of eDNA influent solution. The unshaded area represents the time in which the influent solution as flowing inside the column; the shaded area demarcates the time during which the tubing was changed to eDNA-free water, flushing the column of suspended eDNA.

was put into the system (based on mean influent concentration). The results are summarized in figure 3. We expected eDNA fluxes to be influenced by biofilms, which we predicted would increase particulate eDNA retention [26,42,43]; however, there was no statistically significant difference between eDNA storage in columns with and without biofilm (Kruskal–Wallis $\chi^2 = 0.3333$, d.f. $= 1$, $p = 0.5637$) or for either substrate treatment (Kruskal–Wallis $\chi^2 = 2.0833$, d.f. $= 1$, $p = 0.148$). While our statistical power is low given low experimental replication (two experiments per substrate treatment), as anticipated, S treatments tended to retain more eDNA mass than PG (figure 3), suggesting a filtering mechanism of smaller pore sizes which may trap larger eDNA particles. Again though, we must note that this result was not as dominant as expected and not statistically significant.

While some studies have shown that eDNA degradation can occur quickly (e.g. 27% eDNA loss per 3 h [41]), we found no evidence for degradation in our influent solution over the course of the experiment (linear regression, $p > 0.05$; see electronic supplementary material, figure 1).

Application of mass balance of eDNA outflow and storage suggests that the proportion of eDNA stored in columns was highly variable, ranging from 12% to 98% (figure 3), and varied greatly between treatment replicates within any single substrate (e.g. PG varied between 12% and 71% retention of eDNA within the column). This is further supported by the rapid flushing of detectible eDNA in the outflow once the tubing was placed in eDNA-free water (figure 2*a–d*); eDNA in suspension was quickly flushed from the column, whereas some remained entrapped in the column (figure 2*a–d*). The variable amount of eDNA storage between substrate replicates is consistent with the idea of random particle size distributions, and that eDNA is polydisperse and not well described by a single particle size or material [29,30].

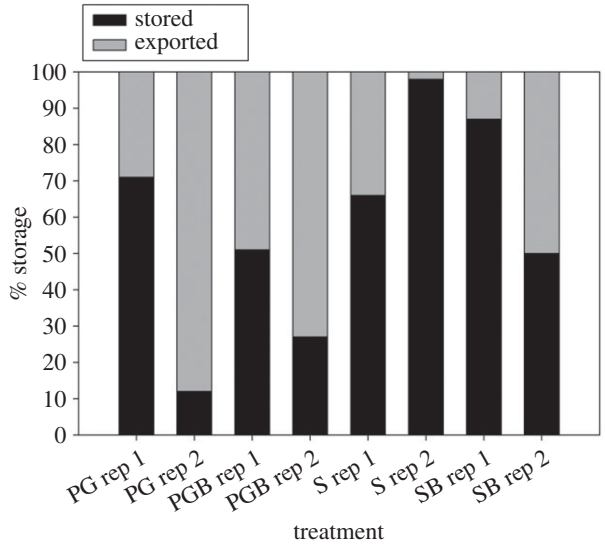

Figure 3. Mass balance of DNA representing proportion of DNA storage (retention) and transport (export) in each treatment for both replicate experiments (1 and 2).

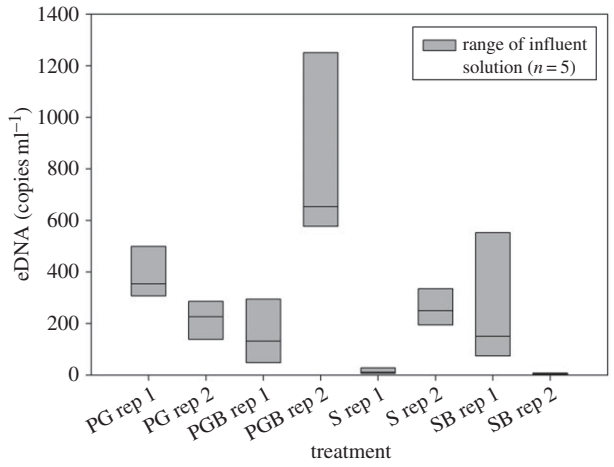

Figure 4. Range of influent solution concentrations ($n = 6$) for each column experiment. Median concentration shown by bar.

3.3. Influent solution

When the eDNA solution was at the quasi-plateau state, eDNA concentrations were highly variable, unlike what one would expect from a classical reactive tracer, and so the origin of such variability must be understood, starting with the influent solution. As noted in the Methods section, we mixed the eDNA solution, which fed the inflow, continuously using a variety of methods, including high flow rates with an aquarium pump installed in the eDNA storage container, which would ensure complete mixing for any reasonable solute or typical particle suspension (accounting for gravitational settling). Additionally, the water taken from the fish tanks had in every case been exposed to the same conditions and exposure duration. However, when we took samples from this influent eDNA laced water, observed concentrations of eDNA varied, in some cases, substantially. The results are presented in figure 4. Although we expected the mean influent solution concentration to remain similar over time and between experiments, we found that the influent solution had no well-defined mean concentration, resulting in different concentrations between experiments (Kruskal–Wallis $\chi^2 = 31.04$, d.f. $= 7$, $p < 0.001$; figure 4). Despite the continuous mixing, it is possible, owing to eDNA's discrete and polydisperse nature [29,30], that sampled concentrations are random, and that the solution is non-homogeneous (i.e. two identically obtained samples from the same 'well-mixed' tank can have different eDNA concentrations). This has potential consequences to field applications of eDNA surveillance [31,32]. Additionally, while fish may be exposed to virtually identical living conditions, the rate at which eDNA is released from them can be highly variable and is likely not deterministic [36].

3.4. Transport parameters

For application of the ADR models, we used the mean influent solution ($\pm$s.e.) concentration as the inlet boundary condition to model a reasonable range of output (figures 5 and 6). Often, the absolute eDNA concentrations did not fall within the expected range based on ADE model output. One feature that the model does appear to capture consistently across all cases is that it estimates a retardation factor of approximately 2, suggesting that eDNA is briefly held back through in the column relative to a conservative tracer; this value appears unchanged whether a biofilm is present or not. Our best-fit model parameters are reported in table 1.

4. Discussion

To begin to understand how eDNA might move through a porous medium, we assumed that it might travel in a fashion similar to a conservative solute or uniformly distributed particle suspension. Therefore, to interpret our experimental data, we applied standard ADR models based on mass balance that incorporate advection, Fickian dispersion and first-order linear adsorption [44]. Using the standard advection dispersion model, our goal was to parametrize characteristic transport properties, including advection, diffusion and dispersion in the way commonly applied to conservative tracers. If eDNA were a monodisperse particle (i.e. one size), one might expect eDNA transport to be well described by such a model [33,34]. While the classical ADE models do provide some useful insights, they do not capture the overall dynamics in a clear and meaningful manner, largely owing to the discrete, heterogeneous and polydisperse nature of eDNA. Given little prior knowledge about the behaviour of macroorganism released eDNA, our results demonstrate that eDNA particles do not behave like a solute or single-sized particles, as it is neither monodisperse nor homogeneously distributed; eDNA particles are polydisperse, with a broad size range and material type [29,30]. The range of sizes spans a substantial range and based on size alone [29,30], smaller particles might behave like solutes and larger particles displaying particle-like dynamics. In fact, given the polydispersity and heterogeneity, it is likely eDNA behaves both dually as a particle and as a solute, requiring that future modelling efforts incorporate such effects.

In addition to the non-uniformity of the particle size in a mixture, eDNA has the added complexity of having non-homogeneous, random distribution of concentration, even when the eDNA solution is vigorously mixed. Our results for the influent solution are consistent with other studies which have shown that even individual organisms in mesocosms can exude eDNA at variable concentrations (e.g. salamanders [15]; fish [36]). In our case, the variability

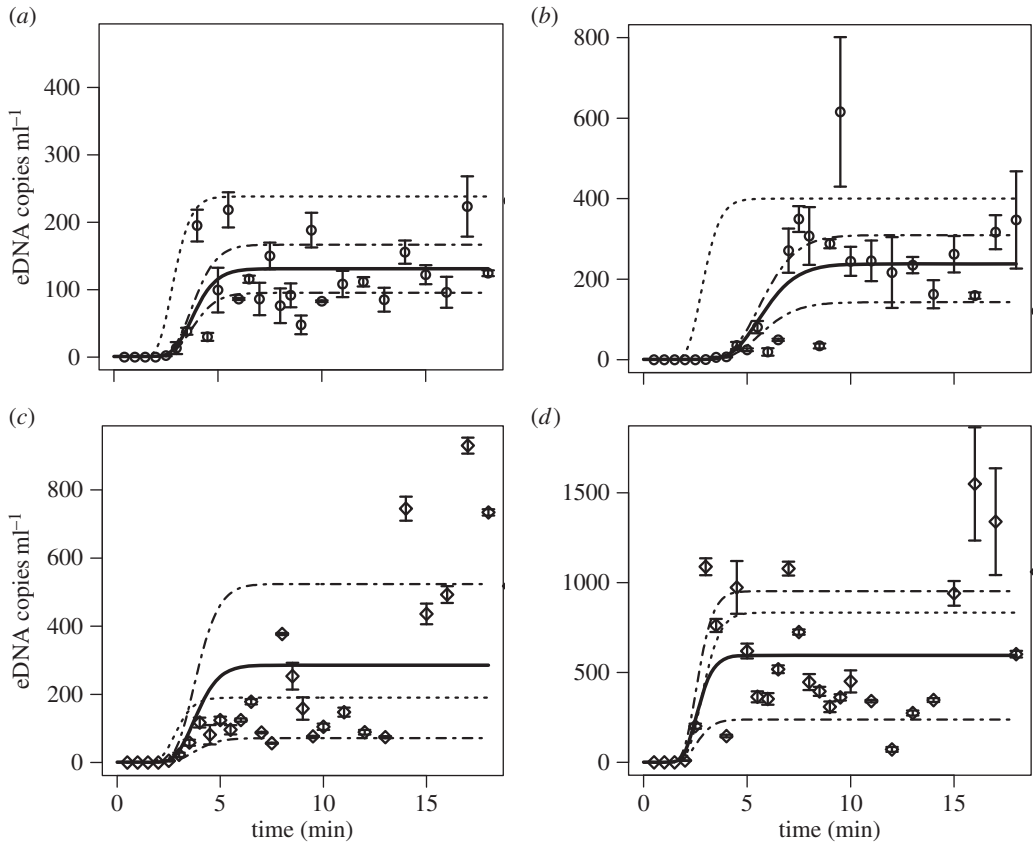

Figure 5. Model results for pea gravel (PG) experiment rising limbs (*a*) PG rep 1, (*b*) PG rep 2, (*c*) PGB rep 1 and (*d*) PGB rep 2 with concentration (copies ml^{-1}, *y*-axis) plotted against time (minutes, *x*-axis). Figures show simulated conservative (finely dashed line, using mean influent solution data), retardation (solid line, using estimated R_f) and minimum and maximum (dashed, using maximum and minimum influent solution with estimated R_f) models. Quantified eDNA concentrations (open dots) in molecules per ml with s.e.m. error bars.

in the influent solution was likely an inherent non-uniformity in concentration. The heterogeneity of initial conditions (i.e. eDNA concentration) complicates modelling and interpretation owing to variable inlet boundary conditions. We found this to be the case repeatedly across each of our experiments here, even though we collected eDNA influent solution from the same collection tank, with the same number of fish, under the same conditions. This result, while unexpected, must be considered when interpreting eDNA detections in field conditions. It suggests that perhaps stochastic models, capable of capturing such uncertainty and heterogeneous dynamics, rather than classical deterministic models, may be more appropriate for eDNA transport studies, the net result being a probabilistic range of eDNA concentrations rather than some deterministic value. Our results suggest that the use of eDNA sampling techniques in the field may necessitate more consideration in sampling effort and modelling to capturing the inherent variability and anomalous behaviour of eDNA particles (CL Jerde, BP Olds, AJ Shogren, EA Andruszkiewicz, AR Mahon, D Bolster, JL Tank 2013, unpublished data), as natural environment variability will likely be larger than in our controlled set-up.

We note that we performed each experiment twice for each substrate and biofilm treatment, and while we recognize that our statistical power is low, we are confident that our observations are an important contribution to sampling design and potential future modelling efforts for eDNA mixtures. While we may not have been able to statistically differentiate any treatment effects between substrate type and biofilm colonization, we did see unique behaviour consistent with a new paradigm supporting that eDNA as a mixture is polydisperse, which makes the use of simplistic models such as the ADE somewhat problematic. Indeed, more work is needed to determine how eDNA moves through porous media and interacts with substrates with varying surface characteristics driving differences in eDNA transport, and we highlight the inherent difficulties in such an effort thus motivating and enabling future efforts both by our group and others. For example, one could perform a separation experiment via serial filtration to determine how eDNA particles of different size fractions may be transported and retained in a complex system. In the experiments reported here, however, we used an eDNA mixture taken directly from a population of fish to best represent the distribution in a natural system. Additional work could better describe how different eDNA material fractions (i.e. cells versus tissue fragments) might be transported and retained within porous media. In this experiment, however, we chose to focus on the entirety of an eDNA mixture, only filtering out material that was too large for the experimental set-up.

We had intended to run additional experiments, but as we collected and analysed the data for each experiment, we questioned whether or not additional experiments would yield more consistent results. We wanted to see if the substrate and variability therein was what gave rise to certain behaviours. To do so, we ran an 'empty' (substrate-free) column to remove the effect of variation in substrate packing and any retention to determine if we would see the same variability in outflow concentrations. In this experiment, the outflow

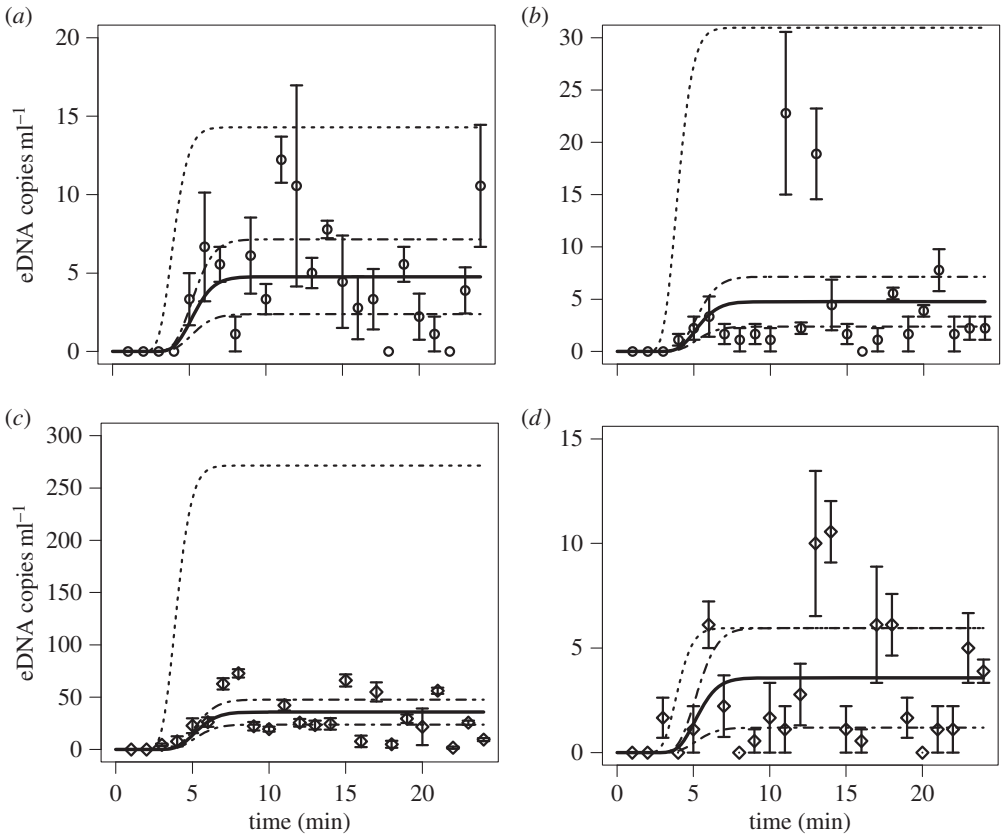

Figure 6. Model results for sand (S) experiments (*a*) S rep 1, (*b*) S rep 2, (*c*) SB rep 1 and (*d*) SB with concentration (copies ml^{-1}, *y*-axis) plotted against time (minutes, *x*-axis). Figures show simulated conservative (finely dashed line, using mean influent solution data), retardation (solid line, using estimated R_f), and minimum and maximum (dashed, using maximum and minimum influent solution with estimated R_f) models. Quantified eDNA concentrations (open dots) in molecules per mL with SEM error bars.

Table 1. Estimated parameters used in final model and flux statistics. Residual sum of squares (RSS) is log transformed for data non-normality.

	parameters						
treatment	$A = v_w$ (cm s^{-1})	D (cm^2 t^{-1})	R	k	mean flux (molecules per minute)	flux RSS	residual variance
PG	0.42	0.015	2.0	0.75	110	2.544	0.150
	0.42	0.015	1.7	0.5	204	5.434	0.418
PGB	0.42	0.015	1.4	0.83	123	7.443	0.438
	0.42	0.015	1.2	1.1	253	8.075	0.475
S	0.42	0.01	2.0	0.75	6	10.868	0.679
	0.42	0.01	2.0	0.73	120	14.949	0.934
SB	0.42	0.01	2.0	0.74	15	13.178	0.824
	0.42	0.01	2.0	0.75	1.2	14.473	0.905

showed a similar stochasticity in concentration that we saw in the packed columns, which convinced us that the variable results yielded from the substrate packed columns were not artefacts, and as such, were a consequence of polydispersity and non-uniformity of the eDNA (electronic supplementary material, figure S2). This result is critical and represents a novel find that is a unique contribution to the literature: variability of this kind makes designing eDNA experiments challenging as adding more and more replicates does not necessarily ensure a convergence to a meaningful and predictable behaviour. In this experiment, additional replication might converge on some mean behaviour, but that is not representative of a real system where the variability must be recognized and embraced.

The idea of polydisperse solutes is by no means new. Natural organic matter (NOM), ubiquitous in natural environments, is well known to be polydisperse, being made up of molecules with broad ranges in size and molecular weights. While similar work has been done studying the effect of polydispersivity on transport and how best to model it in the context of NOM [45–47], it offers simpler interpretation as while it is polydisperse, it can typically be treated as homogeneous; that is, in

a well stirred bucket samples of NOM will all yield very comparable concentrations [25]. Additionally, the size distribution in NOM is such that most molecular weights fall into the range where they can be well described as a solute [47]. In contrast, the range of sizes associated with eDNA suggest that the smallest fractions might behave as a solute, while the largest may not be categorized as such, and thus we propose the terminology 'solute–particle duality'. To address this issue and build more reliable models, further study of eDNA transport and retention will be needed in order to refine our understanding of modelling the unique particle-solute duality exhibited by eDNA particles. For clarification, we note that by the term 'particle-solute' duality we do not mean that an individual eDNA particle will display the behaviours of both, but rather that a solution of eDNA will display characteristics of both.

Additionally, we found that eDNA is retained in a packed column regardless of substrate size and presence or absence of biofilm. We suspect that this is due to reversible sorption of certain size fractions of eDNA on to the porous substrate, as reflected by a retardation coefficient of approximately 2 in the column BTC interpretations. Retardation typically reflects reversible (i.e. rapid/instantaneous) adsorption/desorption. Additionally, other size fractions, likely larger particles, appear to get caught in the porous medium or on biofilm, either permanently or at least over the timescales of our current experiments with less mass breaking through the system than being injected into it, although the fraction of mass retained varied significantly from experiment to experiment with no clear and identifiable control on what drove this behaviour. Similar results have been shown in sand-packed columns for monodisperse particles such as *Cryptosporidium* oocysts [48] and for *E. coli* [49]; however, eDNA that is shed from macroorganisms is a particle with a wider range of sizes and sources than noted in the microbial transport literature [29,30]. In another context, organic particles have been shown to slowly release after initial deposition [49,50]; for the case of NOM, which is also polysdisperse, but more homogeneous than eDNA, different size fractions are adsorbed and released at different rates [45] leading to a broad distribution of travel times and models that reflect these broad distributions appear capable of describing their anomalous behaviour [46,47].

Understanding how biological materials are transported through aquatic systems is a significant challenge in environmental science. Because the size of aquatic particles helps determine their characteristics and interactions with other particles and the environment, our results demonstrate that transport models for eDNA must be informed by the particle size distribution of target species eDNA to maximize detection probabilities and interpretations of what such a detection means. For example, in otherwise virtually identical streams, the presence of a substrate and its specific makeup can significantly impact transport processes [27,50], as can biofilm [42,43]. Specifically, the presence of a substrate usually gives rise to a broad range of transport velocities, with fast flow in the open channel and slow flow through the underlying substrate, resulting in broad travel time distributions that are not readily captured by conventional modelling strategies, even for conservative tracers. The structural makeup of the substrate controls the characteristics of this travel time distribution [51]. Such effects are likely to also impact eDNA transport.

From the perspective of conservation biology, our results do not paint the picture we had hoped them to portray, that is that eDNA transport can be described using conventional transport models and that thus detections of eDNA can be backtracked and implemented in an inverse model to identify where fish populations might be located. Rather they show that eDNA transport is significantly more complicated than that for a conservative or simple reactive tracer and that eDNA displays multiple levels of complexity that standard modelling approaches, at least in their current forms, simply cannot capture. That being said, our data do identify certain key behaviours, which should be accounted for in future model development including the inclusion of behaviour consistent with polydisperse mixtures and the incorporation of non-uniformness of mixtures.

5. Conclusion

Techniques measuring eDNA from rare species in streams and rivers are providing a platform for improved species management [2]. Detection using eDNA will likely be a useful tool to augment conventional and targeted sampling efforts to confirm species presence and can be used to inform and guide intensive sampling in the vicinity of a positive eDNA detection. However, many questions remain about what it means to detect eDNA of a given species in flowing waters, especially in relation to whether that species is presently nearby or some distance upstream, and when it might have been there in the past, which poses challenges regarding mechanisms that allow for a species to be detected with eDNA methods under natural conditions.

Our results suggest that eDNA is polydisperse with no well-defined continuous concentration being shed into the environment. While our initial approach was to treat eDNA as a solute, and therefore, we attempted to apply well-known simple and traditional transport laws to study its movements, our results suggest that eDNA mixtures in water are neither uniform nor monodisperse; rather, eDNA behaves dually, showing behaviours characteristic of pure particle suspensions as well as of pure solutes, complicating interpretation and predictive modelling significantly. The nature of our observations suggests that a more stochastic approach to eDNA modelling and field sampling design is merited to represent the inherent stochasticity of eDNA mixtures.

Ethics. Our research presented has been approved by the Institutional Animal Care and Use Committee (permit no. 16-015, 'Development of an Environmental Metagenomics Approach for Monitoring Aquatic Biodiversity') of the University of Notre Dame (USA).

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. D.B. and J.T. conceived of the study. All authors participated in collecting field data and writing of the manuscript. A.S., E.A. and B.O. carried out the molecular laboratory work and data analysis. A.S., E.A. and D.B. carried out the statistical analyses and modelling efforts. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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