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Water Flow and Biofilm Cover Influence Environmental DNA Detection in Recirculating Streams

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Environmental Measurements Methods

Water flow and biofilm cover influence environmental DNA (eDNA) detection in recirculating streams

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Title: Water flow and biofilm cover influence environmental DNA (eDNA) detection in recirculating streams

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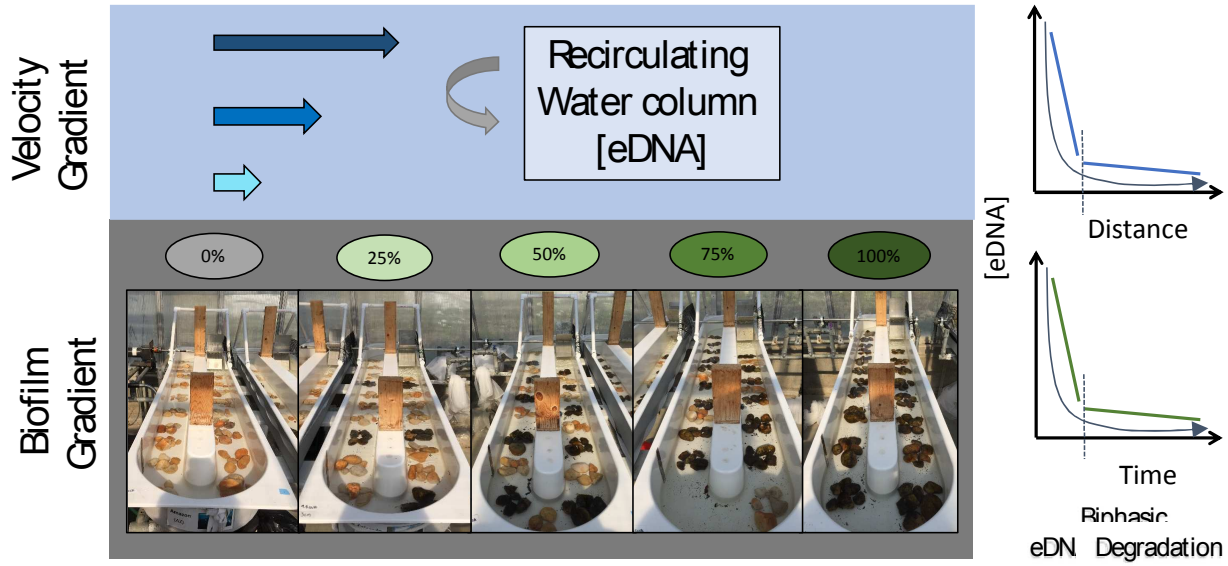
Abstract:

The increasing use of environmental DNA (eDNA) for determination of species presence in aquatic ecosystems is an invaluable technique for both the ecology as a field and for the management of aquatic ecosystems. We examined the degradation dynamics of fish eDNA using an experimental array of recirculating streams using a "nested" primer assay to estimate degradation among eDNA fragment sizes. We introduced eDNA into streams with a range of water velocities (0.1-0.8 m s^{-1}) and substrate biofilm coverage (0-100%), and monitored eDNA concentrations over time (~10 d) to assess how biophysical conditions influence eDNA persistence. We found that the presence of biofilm significantly increased initial decay rates relative to previous studies conducted in non-flowing microcosms, suggesting important differences in detection and persistence in lentic vs. lotic systems. Lastly, by using a nested primer assay that targeted different size eDNA fragments, we found that fragment size altered both the estimated rate constant coefficients, as well as eDNA detectability over time. Larger fragments (>600bp) were quickly degraded, while shorter fragments (<100 bp) remained detectable for the entirety of the experiment. When using eDNA as a stream monitoring tool, understanding environmental factors controlling eDNA degradation will be critical for improving eDNA sampling strategies.

Key Words: eDNA, degradation, nested primer assay

Graphical Abstract



Introduction:

Modern conservation science and natural resource management are in an era of rapid transformation, facilitated by a growing molecular "toolbox" of approaches that enable the identification of targeted species in environmental samples without direct observation [1–3]. The emergence of modern molecular techniques for inference of species presence in ecology has been bolstered by the development and application of environmental DNA (hereafter, eDNA) methods for many species in aquatic systems [4]. The eDNA approach uses genetic material captured and identified from water samples, and can include free-floating extracellular DNA, feces, tissue, and other excretions and sloughed materials [2,4,5]. The rapidly expanding use of eDNA techniques towards direct applications, including species monitoring and management, is directly related to the higher sensitivity of the approach relative to conventional sampling methods such as netting, seining, and snorkel surveys [6]. Despite the growing use of eDNA for

invasive, rare, and endangered species monitoring applications [7–11], methodological testing is still required to determine the inferences that can be drawn from eDNA detection in varying aquatic systems. This may include the standardization of eDNA sampling strategies and sampling design [12], in addition to recognition of what environmental factors influence detectability in space and time.

Importantly, optimizing eDNA techniques in flowing aquatic systems has significant potential to move the technique towards applications beyond presence/absence information [13], which is the ultimate goal for both monitoring rare species and for the management of invasive species [14,15]. First, these applications must be sensitive enough to detect species reliably when they are rare or in low abundance, which can result in false negatives [16,17]. Second, these methods need to account for flow and environmental conditions, as eDNA signals become diluted as they are transported downstream and the probability of positive detection decreases [18]. Additionally, determining the precise source of eDNA in flowing systems is challenging due to the combined effect of downstream transport [18] and eDNA degradation [5,19–23], which alter eDNA concentration in the water column after it is released from an organism [24–26]. Moreover, rapid degradation has been hypothesized as a cause of decreased detectability over time, and therefore limits temporal and spatial inference of positive detections; the result is an increased potential for false negative detection when a species is present [20].

In addition to the challenges resulting from transport, there is a stark lack of data that may show how biological factors, such as substrate biofilm colonization, can influence eDNA rate constants in flowing waters. To date, a majority of eDNA degradation studies have been performed in non-flowing mesocosms or standing water such as ponds [5,19,27]. While variation in the biology of the water column has been shown to influence rate constants [19], there have been

no previous studies investigating the influence of benthic biofilms on eDNA degradation in flowing waters. Importantly, the presence of biofilms strongly influences the retention of nutrients, carbon, and particles during downstream transport [28–32], and the inherent properties and physical structure of biofilms can entrap eDNA and may promote *in situ* decay [30].

In this study, we assess the impact of biological (i.e., biofilm) and physical (i.e., water flow) stream characteristics, and their interactions, on the degradation rate coefficients of eDNA. We set up artificial recirculating streams with varying biofilm coverage, introducing known quantities of fish eDNA into recirculating artificial streams to measure the decline in eDNA concentration over time. Our goal was to address two key questions: 1) *Does water velocity, which ranges considerably in natural streams, influence eDNA rate constants?* and 2) *How does the presence of benthic biofilms alter eDNA rate constants*? We hypothesized that lower flow velocities would increase eDNA removal from the water column due to the increased potential for settling, while conversely, higher velocities would promote either continued suspension or resuspension of eDNA particles during recirculation. While these measures might not influence the true rate of eDNA decay, they would be included in the overall removal of eDNA from detection and would thus influence how far downstream eDNA might potentially travel. We expected that increased biofilm coverage would also increase eDNA removal rates, due to increased retention and processing. In most studies to date, only uncolonized substrate has been considered, but it is known that streambed surfaces, and their associated biophysical complexity, can significantly impact retention of eDNA [18,30]. Additionally, we expected that rate constant would depend strongly on eDNA fragment length, and thus we applied a "nested primer" approach [33,34] to assess rate constants of varying fragment length in recirculatinsg streams. While such an approach has been used for estimating fish biomass [34] and for assessing eDNA

106 degradation in water tanks [33], we empirically tested the utility of fragment length as a result of
107 primer choice under the presence of flow and varying biofilm coverage.
108
109 **Methods**
110 *Site Description*
111 We conducted a 10-day experiment in August 2015 using 15 artificial streams
112 constructed of composite fiberglass, which were housed in a greenhouse at the Cary Institute of
113 Ecosystem Studies, Millbrook, NY. Because we wanted to isolate the influence of substrate
114 biofilm and velocity, we kept all physiochemical factors such as water pH, temperature, and
115 nutrient concentrations consistent among streams. We allocated the artificial streams along two
116 experimental gradients: velocity (Low = 0.1 m s^{-1}, Intermediate = 0.5 m s^{-1}, High = 0.85 m s^{-1})
117 and the proportion of rocks colonized with biofilm, relative to uncolonized rocks, at 5 levels (0,
118 25, 50, 75, 100%). For the biofilm treatment, three months prior to our experiment, we deployed
119 rocks (cobbles, 5-10 cm diameter quartz Maryland River Rock; Ayers Supply, Clarks Summit,
120 PA) in a riffle–run section of a nearby 4th order woodland stream (East Branch, Wappinger's
121 Creek, NY). After the three-month incubation period, rocks had well-developed biofilm. To
122 establish our biofilm treatment in the artificial streams, we transported colonized rocks to the
123 artificial stream facility in large buckets with a small amount of stream water. We then placed 0,
124 25, 50, 75, or 100 colonized rocks into each stream, adding the appropriate number of bare rocks
125 to ensure that each stream contained 100 total rocks. We placed all rocks in the streams in
126 clusters, with biofilm-colonized surface up to mimic natural stream-bottom conditions. Using
127 rocks taken from the streams within the first 24 hrs, we quantified biofilm (as chlorophyll a and
128 organic matter) using standard methods (see SI Figure 2). For the velocity treatment, one

paddlewheel motor rotated for a block of 5 streams, keeping recirculating velocity constant in that block of streams. We manually set the motors and then used a tachometer to measure the rotations per minute (rpm), converting rpm to water velocity using the angular flow of the paddle wheel (rpm * 2 π r / 60 s = m s^{-1}). After substrate placement and tuning the paddle wheels, we filled each stream with 20 L of low-nutrient groundwater and allowed biofilms to acclimate in the artificial streams for one day prior to eDNA addition.

Experimental addition of rainbow trout eDNA

To determine that the streams did not contain any target eDNA, we sampled the water column for eDNA in each stream after adding biofilm-colonized rocks and prior to adding our eDNA solution, and we found no detectable target eDNA in any pre-experiment water samples. Once the streams had acclimated for 24 hrs, we added 20 L of water with rainbow trout (*O. mykiss*) eDNA to each stream for a total experimental volume of 40 L. We collected our eDNA "release" water from a hatchery pool filled with rainbow trout fry at the New York Department of Environmental Conservation Fish Hatchery in Van Hornesville (NY) and used the solutions within 3 hours of collection. Before addition to the recirculating streams, we filtered the release water through 1 mm mesh to remove any large particles. Prior to addition, we obtained our starting eDNA concentration by sampling the eDNA solution (N=15) using 250 mL bottles, which were immediately filtered and stored (see SI methods).

After adding the rainbow trout eDNA release solution, we collected 250 mL samples from each stream over the course of 10 days, at intervals of 15 min, 30 min, 1, 1.5, 2, 4, 8, 12, 18 hr, and 1, 1.5, and 2 days, and then daily for the next 10 days (n= 20 sampling points per stream). Each time we collected a sample, we replaced the sample volume with 250 mL of groundwater,

and we also replaced the water lost due to daily evaporation using the same low-nutrient groundwater (~1-5 L), to maintain all streams at the same flow volume throughout the experiment. We replaced any lost water (from evaporation or sampling) using low-nutrient groundwater from the stream facility. After sampling, we briefly stored each eDNA sample on ice in coolers before filtering; each sample was filtered within 30 minutes of collection. We tested for eDNA contamination in two ways: we placed five sealed 250 mL sample bottles filled with DI water into the storage coolers and in the lab, we filtered five samples of 250 mL of groundwater from the Artificial Stream Facility. We then processed the cooler and lab blanks in the same manner as stream samples. Detailed protocols for sample filtration and storage, eDNA extraction and quantification, and primer set development can be found in the SI Methods and SI Table 1.

Modeling and Statistical Analysis:

First, in order to confirm the implementation of our biofilm treatments, we used one-way ANOVAs to test for differences among biofilm biomass metrics (i.e., chl *a* and benthic OM), in addition to temperature, dissolved organic carbon, and pH (SI Figure 1).

Then, we measured the eDNA concentration from each sample using three different primers that amplify different fragment lengths (697, 347, and 97 bp) along the same gene, removing data with concentrations below the qPCR limit of quantification determined by our standard (5 copies/mL). Using all data that was above our limit of quantification, we analyzed the change in eDNA concentration data over the temporal sampling sequence using two fitting procedures to determine the best representation of eDNA decay: a single-phase exponential degradation model or a biphasic (two-phase) degradation model. Biphasic degradation indicates

that a portion of material is biologically labile or physically "sticky" and thus removed from detection quickly in the first phase of degradation. The remaining portion is more resistant to degradation [35], and is either more recalcitrant or less likely to become physically trapped in the benthos. To estimate these two rate constants, we confirmed these breakpoints statistically using the package *segmented* [36,37] in R (Version 3.5.0), which optimizes the highest coefficient of determination of each trend line (R^2). Then we fit a line to the first section of data (on a log-linear graph) until break point, t'. The slope of the first line corresponds to k_1 (day $^{-1}$), or the primary rate constant, while the fit of the line after the breakpoint represents k_2 (day $^{-1}$), the secondary rate constant. These two parameters were combined to create a continuous fit equation [19] where C_0 is the initial concentration:

$$C = C(i)\begin{cases} C_o e^{-k_1 t} & if \quad t < t' \\ C_o e^{-k_1 t'} e^{-k_2 (t-t')} & if \quad t \geq t' \end{cases}$$

In some cases, only a single-phase model was necessary as no breakpoint was observed, and the data was fit to a single-phase exponential degradation model: $C = C_0 e^{-kt}$. We also tested for the effect of chlorophyll a and biofilm organic matter on each term (k_1, k_2, and t') using stepwise linear regression analyses.

Then, to compare eDNA degradation between each stream, we also used a linear mixed effects model (abbreviated as LMM) to evaluate the differences in estimated rate constant coefficients among primer lengths and across the factorial treatments using R package *LME4* [38]. The model included both random effect on each stream and fixed effects on the velocity and biofilm treatment, as well as the model intercept. We also tested for interactions, such as bio*vel, bio*time, and vel*bio. Summary statistics can be found in SI Table 3.

Finally, in addition to estimating each parameter, we used a select subset of scenarios to compare both experimental treatments (biofilm x flow) and the influence of primer size (i.e.,

$C(i) = C_o e^{-k_1 t}$) using the k_1 term for the primer amplifying 97 and 697 bp fragments for the four velocity/biofilm treatments from the artificial streams (Low – 0% biofilm, Low – 100% biofilm, High – 0% biofilm, High – 100% biofilm). For each, we modeled the decline in eDNA concentration from 100,000 eDNA copies mL^{-1}, estimating eDNA concentration, based on the predictive model, at times 1, 3, 6, 8, 12, and 24 hrs from the initial release. This starting concentration is high and represents quantities that would come out of a fish hatchery or a densely populated area. To extend our results to what would happen in flowing waters, we also converted the eDNA decay over time to decay over distance using the equation $C = C_0 e^{-kx}$, where x is distance downstream in meters, for both the low and high velocity treatments (0.1 m s^{-1} and 0.8 m s^{-1}).

We performed all modeling and statistical analyses using R Studio (R Version 3.5.0), and summary statistics can be found in SI Table 3.

Results & Discussion

Biphasic degradation is an important "fate" of eDNA

The successful detection of eDNA in the water column is the combined result of production, removal mechanisms, and degradation, and these factors contribute to the sporadic distribution of eDNA in the aquatic environments [20]. Previous studies, such as work by Eichmiller et al. [19] and others [39], suggests that some proportion of aquatic eDNA is likely labile and degrades quickly, while the remainder is more resistant to degradation. Our findings are consistent with these earlier studies as a biphasic exponential degradation model consistently produced a better fit to the eDNA degradation data in all streams and for all fragment lengths (R^2 = 0.72-0.99; SI Table 4), compared to single-phase exponential models. The only exception

occurred when eDNA concentrations declined below detection so rapidly that no breakpoint was observed (SI Table 4). In general, eDNA concentrations declined rapidly in all streams, with 80-90% of the degradation occurring within the first day regardless of primer size (SI Figure 2). After this point, degradation was followed by an abrupt change at the break-point t', leading to a longer, slower rate constant, and the break-point consistently occurred during the first 24 hours (i.e., between 4 hrs-1 day) of sampling for all size fragments (SI Table 2).

Flow had little effect on rate constants, but increased potential eDNA transport distances

We had expected that water velocity would result in either differential settling of eDNA particles (i.e., faster removal in Low treatment) or increased evidence of resuspension (i.e., slower removal in High treatment). However, the rate of primary degradation (k_1) by velocity was unique for each primer length (*697:* High < Low < Intermediate, *455:* Low = Intermediate < High, *97:* High < Low = Intermediate; Figure 1). In contrast to primary degradation, for the secondary rate constant (k_2), we found no consistent pattern among velocity treatments. Using linear models to compare the slopes of the decline in concentration over time, we observed that velocity was a significant variable only in the model for the 455 bp fragment ($p < 0.05$), moderately significant ($p < 0.10$) for 697 bp, and non-significant for the 97 bp fragment, however the full models were not statistically significant for either the 697 or 97 bp fragments. When comparing a stepwise linear regression approach exploring predictors of the resulting degradation parameter estimates (e.g., k_1), velocity was only included in the final model for the 697 bp fragments (SI Table 3), while chlorophyll a and benthic organic matter were stronger predictors for the estimates for the degradation of the 455 and 97 bp fragments. Consistent with this observation but against our initial expectations, we found no statistical evidence of

interaction between our biofilm and velocity treatments on eDNA degradation (LMM, $p > 0.05$; SI Table 3). Thus, we found no consistent influence of the velocity treatments on eDNA degradation among primer sets (SI Table 3) suggesting an overriding influence of biology in driving rates of degradation [5].

While flow itself did not influence our observed rate constants in our experimental setup, in the context of natural streams our results imply that water velocity has strong control on water column eDNA concentrations as downstream flow advects and disperses eDNA downstream. If continuous eDNA degradation and downstream transport is simultaneous, variation in water velocity likely results in spatial separation of eDNA concentration at the same "state" of degradation depending on the instream conditions. This spatial separation has strong implications about the interpretation of water column eDNA concentrations, particularly in relation to whether a species is presently nearby or some distance upstream. In this study, our velocity treatment ranged from 0.1 to 0.8 m s^{-1}, which represents only a small portion of the range in environmental flows and water fluxes found in natural systems. For example, in an inter-biome study, the mean velocity across 36 headwater streams (1st to 3rd order) ranged from nearly 0.01 to 0.93 m s^{-1} ($Q = 2$ L s^{-1}, up to 268 L s^{-1}) [40], which presents a broad range of potential instream velocities within a stream channel. Even within natural systems, the interaction between the water column and the streambed creates variation in water velocities that are likely not captured in these simplified recirculating streams.

Biofilm increased eDNA rate constants

In contrast to velocity (i.e., advection), biofilms had a stronger effect on degradation, with faster rates found in streams with >50% biofilm cover relative to bare or nearly bare

substrate (0 and 25%, respectively, Figure 1). Across all biofilm and velocity treatments, we used stepwise linear regressions to test for biological predictors of degradation estimates for k_1 and k_2. For the 97bp fragment, k_1 was best predicted by biofilm metrics (Full LM: $R^2 = 0.44$, $p = 0.04$), with biofilm chlorophyll *a* ($p = 0.013$) and benthic OM ($p = 0.06$) strongly predicting k_1. Similar effects were observed with the 455 bp fragments (Full LM: $R^2 = 0.38$, $p = 0.08$; chlorophyll a $p = 0.006$; OM $p = 0.067$), but as mentioned, velocity was the only significant predictor for the estimates resulting from the 697 bp fragments (Full LM: $R^2 = 0.38$, $p = 0.08$; Velocity $p = 0.03$). However, for all fragment lengths we found no significant predictors of k_2 and t'.

The specifics of biofilm-mediated eDNA degradation remains unexplored to date, and in our study, we cannot separate the relative role of the water column versus the benthic biofilms on the persistence of water column eDNA. While there is evidence that stream water physiochemical variables influence rate constants, such as temperature, pH, and dissolved organic carbon (DOC) in lakes [19,20,41], these factors did not vary among the recirculating streams used in this study (ANOVA $p > 0.05$ *for all*). While biofilm coverage increased microbial colonization as measured via stream metabolism (Hanrahan et al. *unpublished data*), we did not quantify microbial activity at the substrate scale (i.e., on individual rocks) and as such cannot tie eDNA degradation directly to bacterial carbon demand. Moreover, a recent study suggested that eDNA degradation was not strongly related to bacterial abundance [41]. It is possible that biofilm architecture alone can act physically as a sorptive "sponge" for eDNA retention, resulting in either temporary or permanent removal of particles from the water column [30,42]. Biofilm-mediated retention increases the likelihood of subsequent microbial processing, but the fate of biofilm-trapped eDNA certainly deserves further study, including how different eDNA source material (e.g., urine vs. cells vs. tissue fragments) might be uniquely degraded.

289

290 *Primer length influenced eDNA rate constants and detectability*

291 Overall, we anticipated that shorter fragments would degrade slower than larger
292 fragments. Our initial rate constant coefficients, k_1, ranged from 8-35 day^{-1} for the 697 bp
293 fragment, 8-32 day^{-1} for the 455 bp fragment, and 5-12 day^{-1} for the 97 bp fragment (Figure 1).
294 Our results were consistent with the findings of a previous study [34] where that eDNA fragment
295 length was related to detectability over time. Depending on eDNA fragment length, slopes
296 describing the shorter fragments (97 bp) were different from longer (455 and 697 bp) fragments
297 based on all pooled eDNA concentration data (LM with interaction term fragment*time, $p <$
298 0.05). Our study was the first to address how the combination of biofilm, velocity, and fragment
299 length would alter instream eDNA concentration, it is clear that we have merely scratched the
300 surface of the potential for the application of assays that target varying fragments. While more
301 testing is needed to determine any detection "thresholds" among different fragment sizes, our
302 primer that targeted smaller bp fragment resulted in eDNA degradation rate constants that were
303 half the rate constant for the longer fragment. Importantly, observed variation in eDNA
304 degradation rate constants as a result of target eDNA fragment length imposes a significant
305 methodological challenge for interpreting eDNA results and assay design.

306 Despite the challenge posed, the application of assays similar to those described in this
307 paper may provide a potential opportunity for improved interpretation of positive eDNA
308 detection under natural conditions. Broadly, the result of our nested primer approach suggests
309 that incorporating such analytical techniques into eDNA approaches may allow more
310 information to be gained from a single sample than a single relative eDNA concentration alone.
311 For example, our study was consistent with the results presented by Jo et al. [34] where detection of

a longer eDNA fragment is correlated with recent species presence, and therefore the relationship between long and short fragments can potentially indicate species recency or eDNA processing. Though the application of this technique demands further study and validation, the choice of primers that amplify different sized eDNA fragments could be optimized to support a variety of experimental approaches or even applied in the context of management goals. For example, if the goal is rapid detection of species and the assay efficiencies are comparable, a larger fragment size might yield contemporaneous results, while a smaller fragment size could be used for more general presence and absence surveys. When larger and smaller fragment assays are combined, the nested primer approach could indicate an eDNA "processing continuum" over time (and space in flowing waters), because the concentration of the smaller fragment relative to the larger fragment should begin to dominate as degradation proceeds. If successful, the use of nested primers could be effective in informing the when a target species was present, especially in flowing waters where inference is confounded as a result of simultaneous transport and degradation.

We do note that this method certainly demands further optimization and testing to determine its reliability and ultimate potential. Currently, the body of literature that describes the application of different fragment sizes on eDNA detection is limited, and results of these studies have been mixed. In one study, the authors observed that longer eDNA fragments degrade faster than shorter fragments in mesocosms [34]. In another, also using a "nested" design in fish tanks, workers found that the rate of decay was not dependent on fragment length but rather its origin (e.g., nuclear vs. mitochondrial) [33]. We would also like to recognize that while in need of further study, the application of dual assays that reliably amplify different target lengths could serve as

334 an additional validation check for eDNA analyses, potentially lending insight on detecting type I
335 and II errors for environmental samples.
336
337 *The importance of degradation rate reporting and modelling*
338 Our results also underscore the importance of reporting eDNA degradation rates under
339 both monophasic and biphasic conditions, so that a more complete picture of eDNA degradation
340 can be drawn. Under all of our experimental conditions, our initial rate constant for the biphasic
341 model, k_1, for eDNA was much higher than previously observed rate constants for mesocosm
342 experiments that reported monophasic decay (k = 0.05-17.9 day^{-1}) and for our estimates of
343 monophasic degradation using data from this study (k = 0.36-2.6 day^{-1}) (Figure 2), though this
344 observation represents an inherent assumption of the biphasic model. While monophasic
345 degradation does capture the decline in eDNA concentration over time, we argue that the
346 significance of the two-phase degradation model is that it more accurately represents the initial
347 rapid decline in concentration that ultimately alters the available eDNA for capture and
348 detection.
349 To this end, we illustrate the importance of interpreting eDNA degradation rates under
350 varying conditions using a simple modeling scenario. To determine how long eDNA remains
351 detectable in the water column, we used the decay curves predicted by a single-phase decay
352 model to reflect how far eDNA might travel in time and over stream distance. These simple
353 models reflect our measured degradation constants under different conditions and thus illustrate
354 the impact of both biofilm and velocity for altering predicted eDNA instream concentrations.
355 For example, after 8 hours the 97 bp fragment degradation curves are distinct: the modeled water
356 column eDNA concentration High velocity – 100% biofilm and Low velocity – 0% biofilm

models differed by 7 times, with "sampled" concentrations of ~4000 copies mL^{-1} vs. ~27,000 copies mL^{-1}, respectively (Figure 3). If these decay rates per unit time were converted to a per unit distance, we see a 10x difference over *space* after only 8 hr simply due to differences in flow (Figure 3). These effects were even more pronounced using the longer primer length (697 bp), where eDNA became undetectable after ~6 hrs, and ~2-10 kilometers downstream. In every scenario, even with a low velocity and high rate constant (Low – 100% biofilm), water column eDNA remained detectable for long distances in high flows (e.g., up to 8 km) before becoming undetectable in the water column.

Context-dependency of eDNA degradation is a challenge for the use of eDNA in flowing environments

Untangling the physical and biological factors influencing eDNA degradation and removal from the water column will improve predictive power and interpretation of eDNA detection results for the presence of critical species in streams and rivers. While the future of eDNA technology remains bright, the leap to estimating species location and abundance in flowing waters remains challenging. Our results suggest that the interaction between biofilm cover and water velocity may further confound attempts to infer target species abundance or location using eDNA approaches. Not only can eDNA be transported long distances in streams and rivers with high water velocities, but eDNA also degrades while in transport, either via water column or benthic process, or the interaction of the two. As such, eDNA detection and persistence depends strongly on environmental context (Figure 1). Moreover, the importance of physical and spatial variability of biofilms, and their effect on eDNA degradation and removal, remains largely unexplored. In the simplified recirculating streams used in this study, the

presence of benthic biofilm strongly influenced the degradation of eDNA; however, biofilms in natural systems are spatially and temporally heterogeneous [29,43], and thus their role in the context of eDNA detection in the field deserves further exploration.

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Supplemental Information

Detailed methods for eDNA quantification, and figures and tables reporting rate constants can be found in the Supplemental Information.

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Ethics

We declare no ethical considerations.

Competing interests

We have no competing interests.

Author contributions

All coauthors equally contributed to the conception and design of the experiment, acquired the data, interpreted and analyzed the data, drafted and revised the manuscript, and gave approval of the manuscript for publication.

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Figures

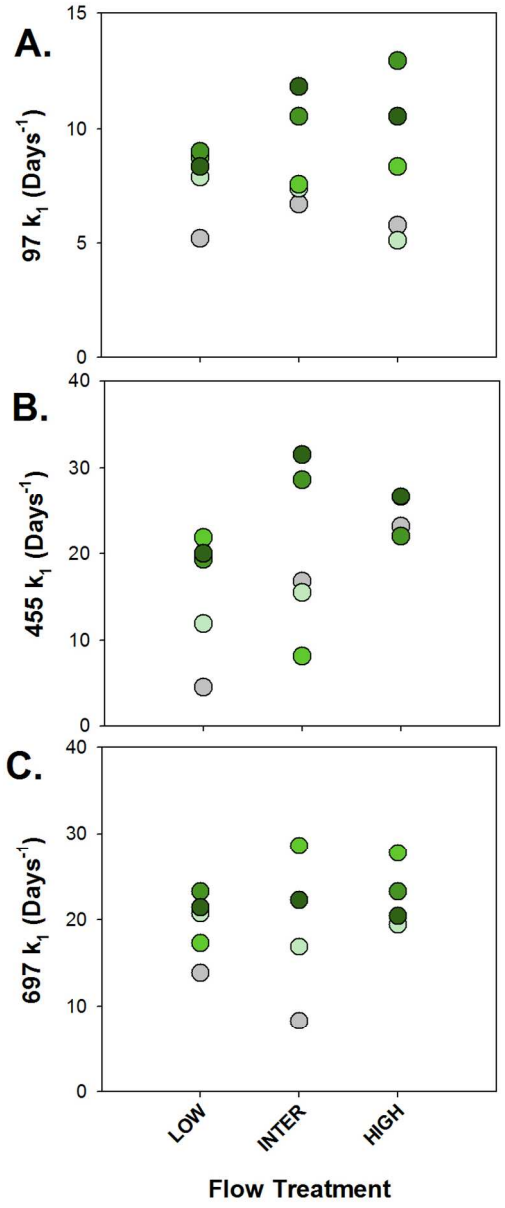


Figure 1: Estimated primary (k_1) degradation terms from biphasic model fitting for: A) 97 bp, B) 455 bp, and C) 697 bp eDNA fragments across all velocity treatments (x-axis). Color gradient of dots represents biofilm cover treatment, from gray (0%) to dark green (100%). Secondary degradation terms are reported in SI Table 2.

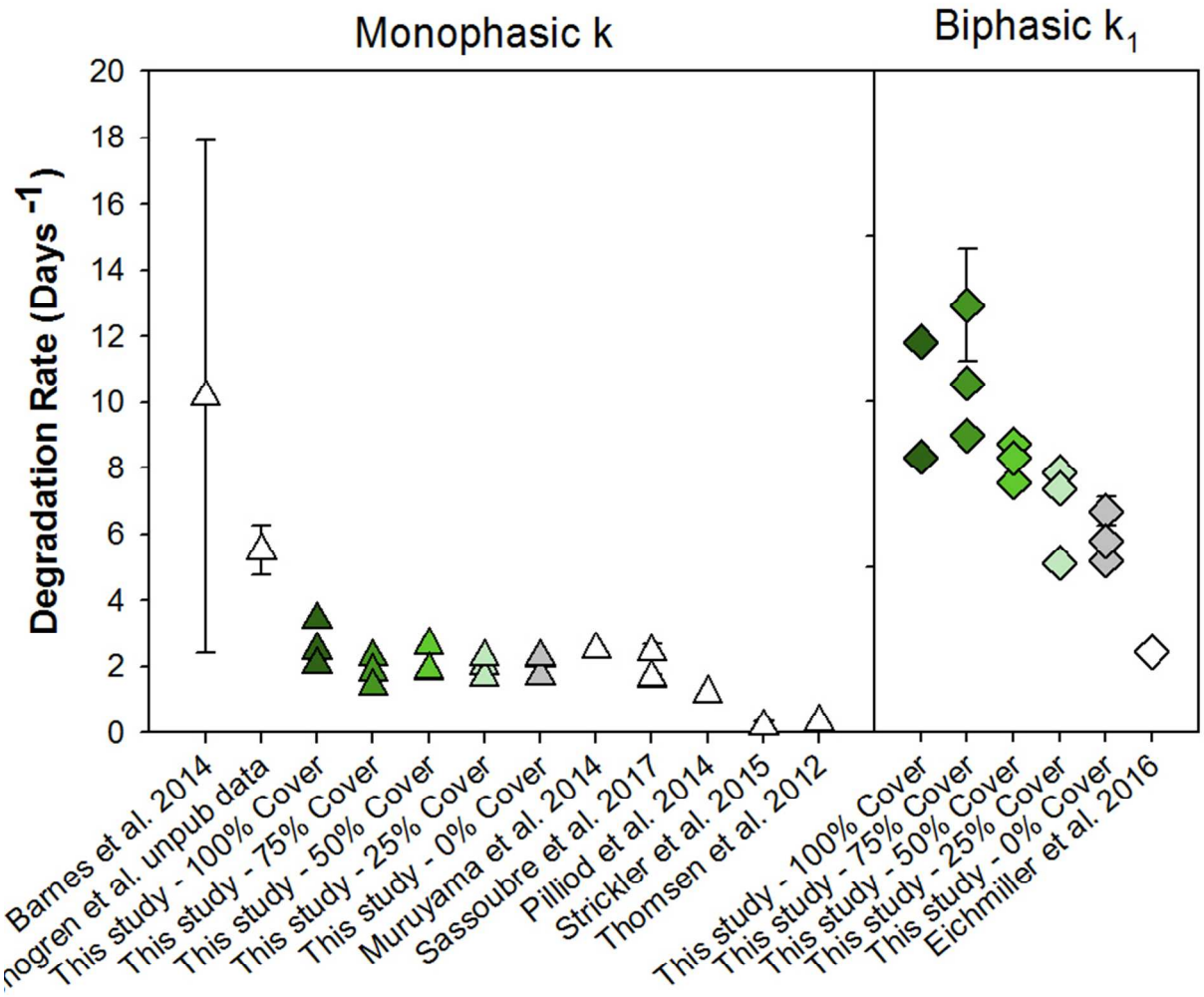


Figure 2: Meta-analysis of previously published studies on fish eDNA rate constant coefficients (white) and this study (gray to green gradient) for monophasic (triangles) and biphasic (diamond) rate constants (k vs k_1). Rate constants expressed in $days^{-1}$ (±SE if reported).

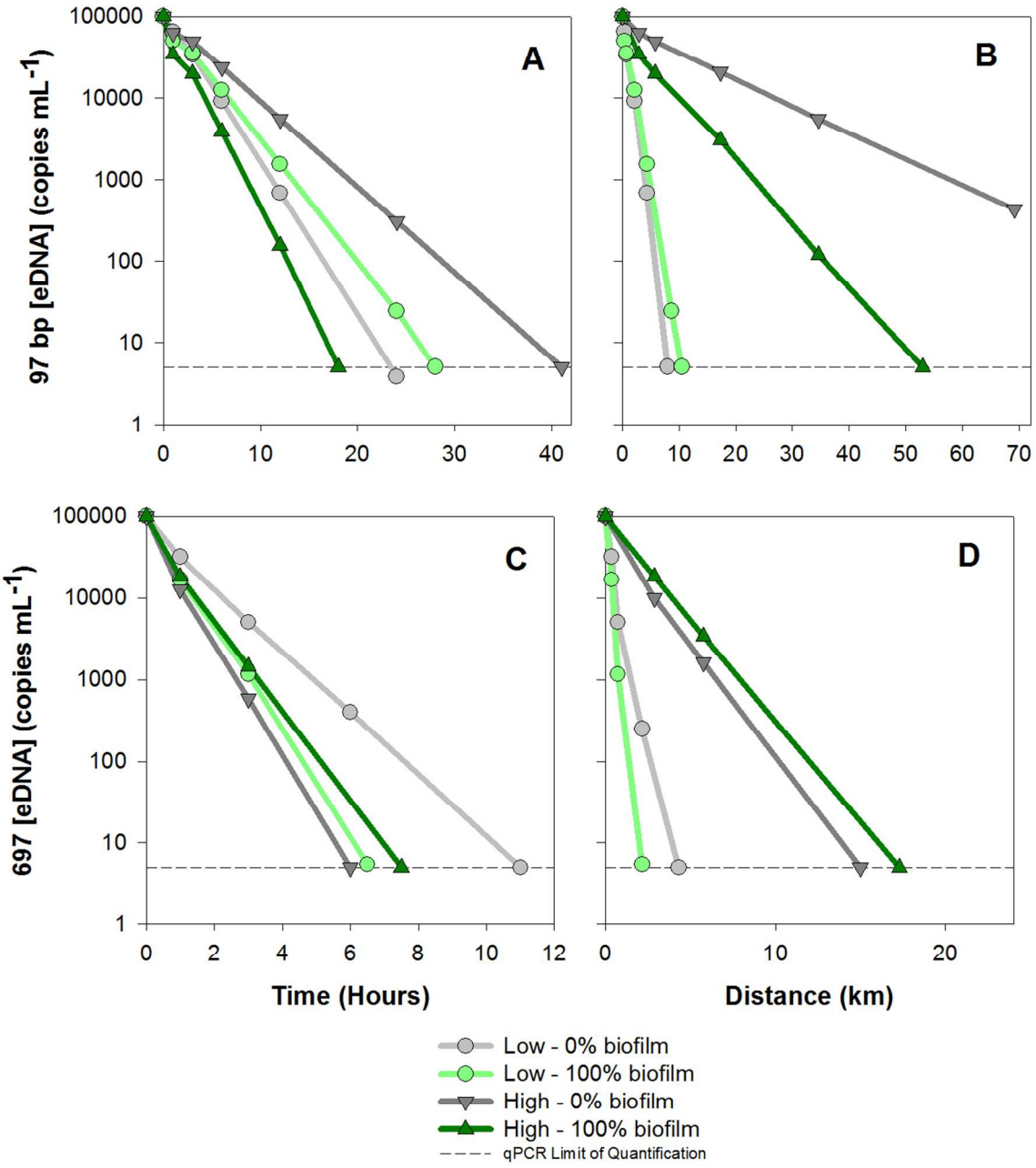
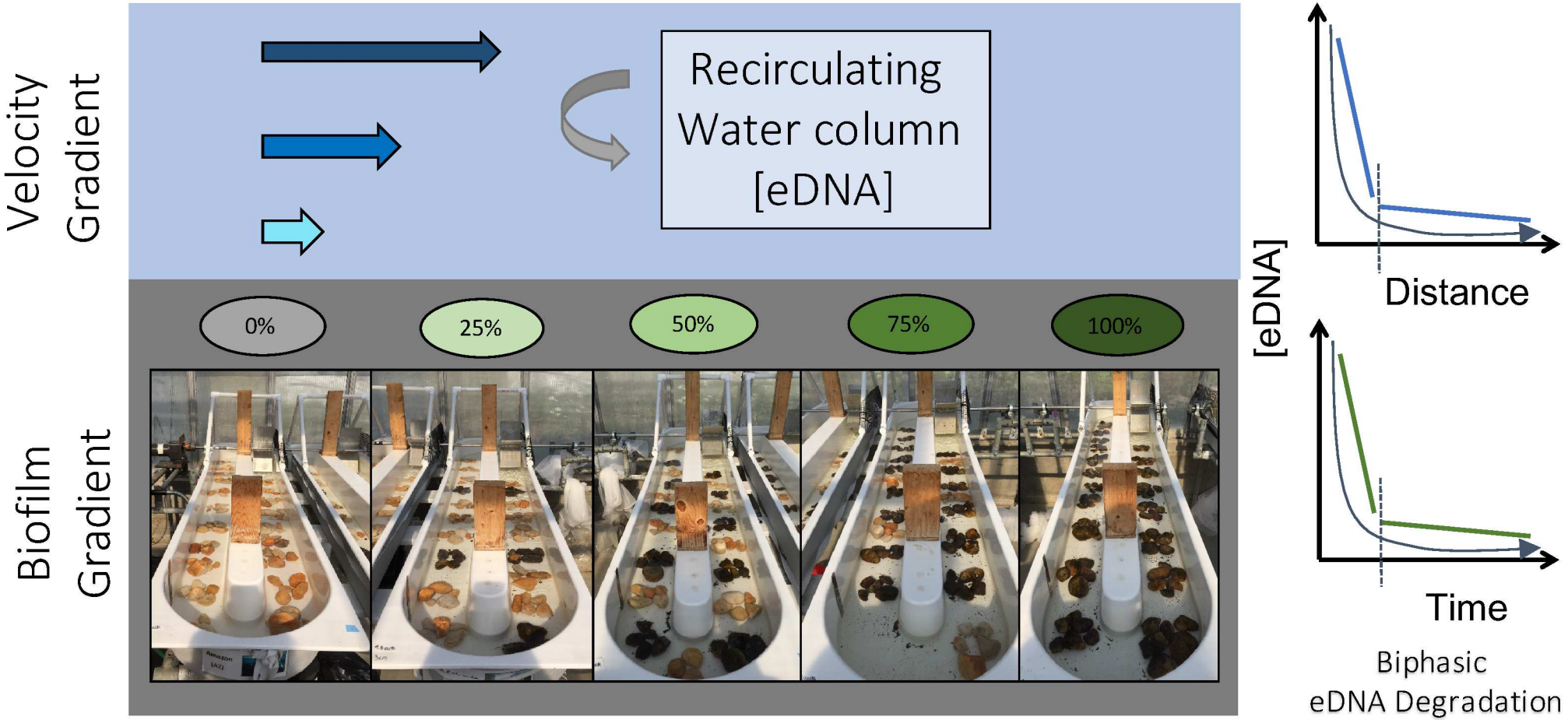
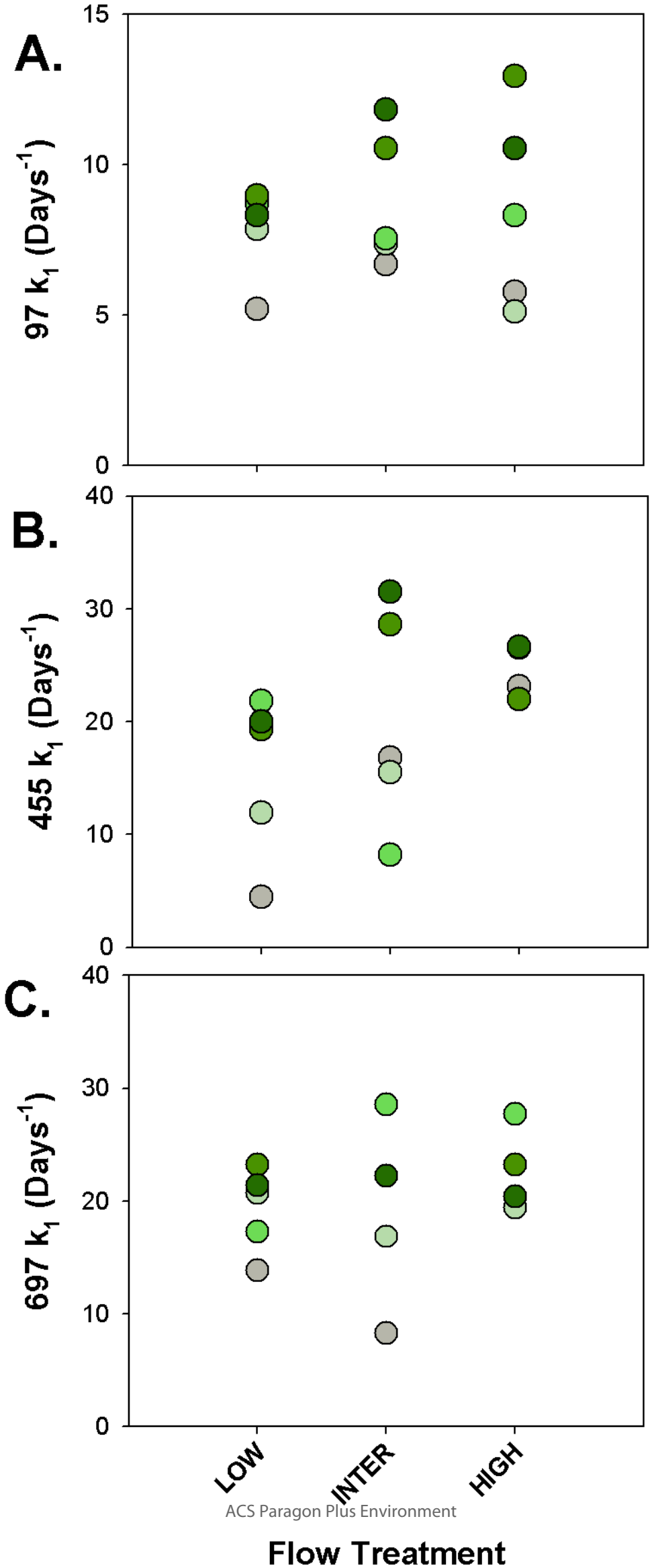


Figure 3: Projected (i.e., modeled) eDNA concentration in time (A: 97 bp fragment, C: 697 bp fragment) and distance (B: 97 bp fragment, D: 697 bp fragment) based on 4 flow/biofilm scenarios (Low-0%, Low-100%, High-0%, and High-100% biofilm coverage) using the estimated k_1 term from the artificial stream experiment.


Velocity Gradient
Recirculating Water column [eDNA]
Biofilm Gradient
0%
25%
50%
75%
100%
[eDNA]
Distance
Time
Biphasic eDNA Degradation


A.
97 k1 (Days-1)
15
10
5
0
B.
455 k1 (Days-1)
40
30
20
10
0
C.
697 k1 (Days-1)
40
30
20
10
0
LOW
INTER
HIGH
Flow Treatment