Riverine distribution of mussel environmental DNA reflects a balance among density, transport, and removal processes

Arial J. Shogren | Jennifer L. Tank | Scott P. Egan | Diogo Bolster | Tenna Riis

Abstract
1. Sampling water for environmental DNA (eDNA) is an emerging tool for documenting species presence without direct observation, allowing for earlier detection and faster response than conventional sampling methods in aquatic ecosystems.
2. However, current understanding of how eDNA is transported in streams and rivers remains imprecise, with uncertainty of how the unique transport properties of eDNA may influence the interpretation of a positive detection. To test the utility of eDNA sensing in flowing waters, we compared quantitative eDNA analyses to zebra mussel density surveys in a Danish river.
3. Although flowing water complicates the relationships between eDNA production, transport, and removal, we found weak but positive relationships between eDNA concentration, zebra mussels, and biophysical parameters. For example, while zebra mussel densities were only moderately predicted by eDNA concentrations, eDNA was most strongly influenced by nutrient concentrations and water velocity. These results may be used to inform future sampling strategies, where hydrological variables could better constrain eDNA fate.
4. We also modelled estimates for net eDNA transport, retention, and degradation to estimate the relative importance of these processes for removing eDNA from the water column. In our study system, physical retention accounted for c. 70% of removal when compared to degradation alone, making it an important process to consider when assessing downstream eDNA transport.

KEYWORDS
eDNA removal, eDNA techniques, eDNA transport, invasive species, zebra mussels

INTRODUCTION

Aquatic species are notoriously difficult to detect with traditional manual capture methods (e.g. electro-shocking or netting) (Lodge et al., 2012; Nathan, Jerde, Budny, & Mahon, 2014). Alternatively, traces of environmental DNA (eDNA) can be sloughed from an organism into aquatic ecosystems and remain in suspension. This genetic material can be captured in a water sample, revealing the presence of a target species (Darling & Mahon, 2011; Ficetola, Miaud, Pompanon, & Taberlet, 2008). The detection and quantification of eDNA offers a novel species detection platform that improves the ability to detect and monitor the distribution of freshwater populations (Rees, Maddison, Middleditch, Patmore, & Gough, 2014). To date, the species-specific eDNA approach has been used extensively to detect the presence and/or absence of species (Jerde, Mahon, Chadderton, & Lodge, 2011; Laramie, Pilliod, & Goldberg,
2015; Mahon et al., 2013; Nathan et al., 2014). The literature demonstrating the successful detection (i.e. presence/absence) of aquatic species has been rapidly growing each year (Roussel, Paillisson, Tréguié, & Petit, 2015), as the ability to detect rare, endangered, or invasive species is of clear value for managers (Kelly et al., 2014; Lodge et al., 2012). While presence/absence surveys can provide inherently useful information, knowledge of species presence alone is limited in its utility. Presence and absence data do not characterise organismal density or persistence in a given habitat, nor provide a precise location for targeted action or management (Hinlo, Furlan, Suitor, & Gleson, 2017; Nathan et al., 2014). Recently, the use of targeted eDNA sampling has begun to move beyond estimating only presence/absence, with increasing interest in understanding how quantitative measures of eDNA (i.e. concentration) relate to organism abundance or biomass.

Quantitative eDNA analyses provide an exciting opportunity to move beyond species presence and absence, as the technique may provide information about the relative abundance of a target species in natural environments (Doi et al., 2017; Iversen, Kiellgren, & Sand-Jensen, 2015; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016). Estimation of species biomass and abundance, based on the eDNA concentration in overlying water, has been successfully conducted in non-flowing tanks, aquaria, ponds, or lakes, with studies finding positive relationships between eDNA and measured density or biomass (Doi et al., 2015; Lacoursière-Roussel et al., 2016; Pilliod, Goldberg, Arkle, & Waits, 2013; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012; Thomsen et al., 2012; Wilcox et al., 2016; Yamamoto et al., 2016). More recently, there have been an increasing number of studies performed in flowing waters (Baldigo, Sporn, George, & Ball, 2017; Deiner, Fronhöfer, Mächler, & Altermatt, 2016; Doi et al., 2017; Pilliod et al., 2013; Rice, Larson, & Taylor, 2018; Tillotson et al., 2018; Wilcox et al., 2016), although estimating species abundance using eDNA concentrations is not always straightforward. Streams and rivers present a significant challenge, as transport, dilution, retention, and resuspension may occur during travel downstream, further complicating the interpretation of eDNA concentration data (Jerde et al., 2016; Pont et al., 2018; Shogren et al., 2017). Several studies have attempted to relate eDNA concentration to species density, and these have had mixed success (Doi et al., 2017; Pilliod et al., 2013; Tillotson et al., 2018; Yamamoto et al., 2016). Some studies have found positive but weak relationships between eDNA and biomass (Carraro, Hartikainen, Jokela, Bertuzzo, & Rinaldo, 2018; Pilliod et al., 2013; Wilcox et al., 2016), while other studies have found no relationship at all (Jane et al., 2015).

Although some correlations in flowing waters have been demonstrated (Thomsen et al., 2012; Carraro et al., 2018), there remain core challenges that must be overcome before informative relative abundance data can be generated in streams and rivers. First, eDNA degradation rates can vary considerably under different biotic and abiotic environmental conditions (e.g. Barnes et al., 2014, Eichmiller, Miller, & Sorensen, 2016; Sansom & Sassoubre, 2017), including temperature (Strickler, Fremier, & Goldberg, 2015), pH (Seymour et al., 2018), sorption (Turner, Uy, & Everhart, 2014), and benthic biofilm colonisation (Shogren et al., 2018). Thus, detection is dependent on the interplay between the density of target species, the amount of DNA released, and variation in rates of dilution and diffusion depending on the environment, temperature, microbial communities, and the rate of DNA degradation. Furthermore, while some studies have validated presence/absence surveys by sampling above and below known populations (Deiner & Altermatt, 2014; Laramie et al., 2015), these studies did not directly address the major factors responsible for moving (i.e. hydrology) and removing (i.e. biology and physical retention) eDNA from the water column. Conversely, the few previous studies that have documented eDNA transport have used either caged specimens as the upstream eDNA source (Jane et al., 2015), or have relied on short-term additions of known quantities of eDNA in small experimental streams (discharge = 1.5 L/s) (Jerde et al., 2016; Shogren et al., 2017). While previous studies have greatly advanced our understanding of how eDNA moves in flowing water, they have lacked the inherent complexity that arises from natural populations that vary in time and space, which may alter the release, distribution, and transport of eDNA in streams and rivers. Moreover, because abundance estimates in flowing waters are not simple, interpreting results from water column sampling will have to rely on significant methodological ground truthing or field calibration. This information must also be paired with modelling approaches that consider biological, physical, and hydrological variables.

To field-test the role of downstream transport and removal mechanisms relative to species density, site selection and environmental context would ideally require three primary features: (1) well-constrained hydrology (i.e. no loss or gain of water) along the river reach; (2) a sessile target species; and (3) known degradation rates to compare the roles of biological and physical removal. In this study, we had a unique opportunity to test the spatial distribution of eDNA relative to local species density of the sessile, and invasive, zebra mussel (Dreissena polymorpha) in a river system in Denmark. The Gudenaa River is a distinctive lake-river catchment system (Andersen, 1994) that has been recently invaded by zebra mussels (Svenningsen et al., 2012), while also being hydrologically well-constrained. As such, the longitudinal gradient of zebra mussel densities served as an opportunity for a natural experiment, and our primary objective was to determine if and how eDNA concentrations were related to zebra mussel densities. Overall, we expected that eDNA concentrations reflect zebra mussel density; however, we anticipated that quantitative relationships would be mediated by riverine characteristics, and therefore incorporating these characteristics would make eDNA transport models more predictive and powerful. We surveyed distributed transects along a 7-km reach of the Gudenaa River (Figure 1), compared quantitative eDNA sampling to a detailed and spatially extensive zebra mussel survey, and estimated relationships between eDNA concentrations, mussel density, and river physiochemical and hydrologic variables. As a secondary objective, we also partition the relative roles of eDNA degradation
and physical retention in a natural river reach. While eDNA degradation reduces concentrations during transport (Shogren et al., 2018), we expected that physical removal processes would be an additional cause of reduced eDNA concentrations. To this end, when zebra mussels were not present (Figure 1), we used simple models to estimate net eDNA retention rates that represent the cumulative effects of physical and biological removal (Jerde et al., 2016; Shogren et al., 2017). We used published mussel eDNA degradation rates (Sansom & Sassoubre, 2017) to estimate the role of physical versus biological removal.

2 METHODS

2.1 Site description

We conducted our eDNA and density surveys in the Gudenaa River in May 2017. We performed two longitudinal sampling surveys of a 7-km reach from Silkeborg Lake to Sminge Lake (Figure 1), sampling longitudinally (n = 32 transects, Table 1). Using a small boat, we performed separate passes to ensure that we could logistically collect the necessary variables, and follow a Lagrangian-style sampling for eDNA. On the first sampling pass, we captured physiochemical variables and performed the density surveys along the entire 7-km reach, noting each sampling location with a GPS. At each transect (n = 32), we took 250 ml grab samples (in Whirlpak bags) to analyze water samples for phosphate as soluble reactive phosphorus (SRP, mg/L), nitrate (NO$_3^-$, mg/L), ammonium (NH$_4^+$, mg/L), seston organic matter as ash-free dry mass (AFDM, mg/L), and seston chlorophyll a (chl a, mg/L) (one sample per transect). We selected three lateral sampling points at river right, mid-channel, and at river left (n = 3 points per transect), and at each, we measured water depth, water temperature, and pH. Then, using a clear bottom bucket so that we could see the stream bottom, we surveyed substrate type (i.e. Fines, Sand, Sand/Gravel, Gravel, Cobble, Gravel/Cobble, Mud, and Zebra Mussel Shells) (EPA & Division, 2004) and dominant macrophyte types (Bowden, Glime, & Riis, 2017). In addition, we used a survey method to estimate benthic substrate, macrophyte, and zebra mussel density, recorded as percent cover (%). On the next sampling pass, we only sampled for eDNA using a Lagrangian-style approach (Volkmar et al., 2011), again sampling at river right, mid-channel, and river left. By floating with the river, with only gentle corrections from an onboard motor, we effectively followed a parcel of water as it flowed downstream. Thus, we captured the physical and biological processes acting on the eDNA within this water parcel. As we floated, we used an extendable water sampler to grab samples at the three lateral sampling points at each longitudinal transect. We describe transect locations, lengths, and characteristics in Table 1.

2.2 Study species

The zebra mussel is a prolific invasive species that has been recently introduced to many areas, including Scandinavia and North America (Gollasch & Leppäkoski, 1999). Zebra mussels have been present in the Gudenaa River system since 2006, and local populations can form large and dense reefs on the sediment surface (Svenningsen et al., 2012). In fact, zebra mussel density in some areas can exceed

**FIGURE 1** Map of riverine sampling transect locations along the c. 7 km reaches from Silkeborg Lake to Sminge Lake. Each dot represents the sampling transect where we performed environmental DNA sampling and zebra mussel density surveys at three points along the transect (n = 3 samplings points per dot, at river right, middle, and river left). White circles represent upstream lake control samples, where we found no observed zebra mussels. Densely populated zebra mussel transects are noted in black (>75% cover) and grey (25–50% cover) circles. We used the transects at the grey squares (zebra mussels 0% cover) for estimation of riverine environmental DNA net retention (as in Figure 4). Hydrolab minisonde locations are noted by stars. RWT, rhodamine WT [Colour figure can be viewed at wileyonlinelibrary.com]
**TABLE 1** Transect characteristics from the Gudenaa River study reach from May 2017 sampling, including: distance from Silkeborg Lake, distance between transects, mean depth, channel width, estimated water velocity at each transect, mean zebra mussel cover (0%-100%), and characteristic substrate at each sampling location

<table>
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<th>Transect #</th>
<th>Distance from lake (m)</th>
<th>Distance between transects (m)</th>
<th>Mean depth (m)</th>
<th>Width (m)</th>
<th>Estimated water velocity (m/s)</th>
<th>Mean zebra mussel cover (% cover)</th>
<th>Substrate transects</th>
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<td>74</td>
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*Substrate key:* F = fines; S = sand; SG = sand/gravel; G = gravel; C = cobbles; CG = gravel/cobble; M = mud sediment; ZM = zebra mussel shells; - = too deep to measure.
>100,000 individuals/m² (Svenningsen et al., 2012). In our study system, these densely populated areas served as our eDNA source. During filter feeding, mussels shed DNA into the environment in the form of sloughed tissue, excreta, and planktonic veligers during reproduction (Henley, Grobler, & Neves, 2006); thus, when only metabolising, filter feeding is the main source of eDNA (Sansom & Sassoubre, 2017). While shell material does contain DNA (Geist, Wunderlich, & Kuehn, 2008), the amount of DNA sloughed from shell material is minimal.

Our central research objective was to understand the physical and biological controls on downstream eDNA propagation from a natural population. While the definition of eDNA can include gametes (mussel veligers 80–200 μm) (Reed, Herod, & Sickel, 1998), the wide size range of eDNA mixtures makes measuring and modelling eDNA transport exceptionally challenging (Shogren et al., 2016; Turner, Barnes, et al., 2014). We attempted to better constrain the already polydisperse nature of eDNA (Shogren et al., 2016) and thus avoided sampling during the time of year with peak veliger production, although this type of sampling would be certainly worthy of further study. We targeted the release of eDNA from filter feeding, and avoided sampling during mussel reproduction in the summer when the water temperature is >12°C (Matthews & McMahon, 1999); instead we sampled in early spring, when mean water temperature was 10.5°C.

### 2.3 Rhodamine release for hydrologic variables

The day before the longitudinal surveys, we performed a pulse addition of a conservative hydrologic tracer (rhodamine WT, RWT). Capturing the downstream travel and dilution of a rhodamine pulse (dilution gauging) is a common method used to measure hydrologic characteristics of the river including travel time and river discharge (Runkel, 2015). To measure the instream concentration of the pulse, we placed logging sensors (Hydrolab Minisonde, OTT Hydromet) at 2,900 m and 6,600 m downstream of the release point to encompass two halves of the river (Figure 1, yellow stars), which we set to measure RWT concentrations every 5 min over a duration of 12 hr. We secured the sensors with rebar in the main flow, suspending the sensors above the streambed, and were confident of complete mixing, having estimated the mixing distance as 600 m based on previous tracer additions. After sensor placement, we added the hydrologic tracer as a pulse by filling five buckets with a known amount of concentrated RWT solution and pouring all buckets simultaneously off the Silkeborg Bridge (Figure 1) that served as our eDNA sampling starting point, achieving a target water column concentration of c. 500 μg RWT/L. From the RWT breakthrough curves at each of these two locations, we estimated effective instream velocity (v, m/s), discharge (Q, L/s), and median travel times (t, hr) using standard methods (Runkel, 2002). We estimated Q as the integral of the breakthrough curve and the initial mass of rhodamine (Moore, 2005); mean v as the Q divided by channel area (A, m²); and travel time as the time to the start of the leading edge of the pulse at each downstream station (Figure 1, yellow stars).

### 2.4 Visual and grab macrophyte and substrate surveys

Given the significant role that both macrophyte beds (Horvath, 2004) and substrate type (Aubeneau, Hanrahan, Bolster, & Tank, 2016) can play in particle retention, including eDNA (Shogren et al., 2017), we surveyed our entire study reach for percent cover and type of both macrophytes and dominant substrate type. At each transect, we sampled three points (A–C) along a lateral transect, capturing the side on river right, the centre of the channel, and the side on river left (Table 1). To give more detail, at each of these sampling locations, we estimated substrate type (Bain, Finn, & Booke, 1985), macrophyte type and cover, and zebra mussel density using both visual estimates and substrate grab samples using a sewage cleaner grab (13-cm diameter) attached to a 2.5-m pole. Substrate type was divided into six categories (Table 1). Macrophyte cover was divided into five categories: 0%, 25%, 50%, 75%, and 100% cover, and macrophyte species were identified to genus visually. Zebra mussel density was documented similarly, with both live and dead zebra mussels counted in the percent cover estimates. We estimated depth at each location using a depth probe (Plastimo Echotest II), and measured channel width and distance between transects using Google Earth (version 7.3.0) from GPS coordinates. Methods for field sampling of physiochemical variables, such as soluble nutrients, chl a, and organic matter can be found in the Supporting Information.

### 2.5 Field sampling for eDNA

We sampled longitudinally, with distances increasing between transects as we travelled further downstream from Silkeborg Lake (0 m). Using a Lagrangian-style sampling method (Kobayashi & Iwata, 2017; Volkmar et al., 2011), in which we follow a water parcel as it moved downstream over the natural gradient of zebra mussels (i.e. the eDNA source), we examined the decline in concentration as we travelled further from dense zebra mussel beds. As we floated downstream, we collected three 500 ml surface water samples from each site along each transect (n = 90 samples total). In addition to the 32 transects capturing the spatial variation laterally across the river, we also sampled for zebra mussel eDNA in Silkeborg Lake (n = 3) and in and below Sminge Lake (n = 4; Figure 1). We used new 500-ml Whirl-Paks® for sample collection, placing samples into pre-cleaned and bleached coolers (Carim, Mckelvey, Young, Wilcox, & Schwartz, 2016). We collected our samples at increasing downstream distance intervals, at the same locations as the substrate, macrophyte, and zebra mussel surveys (Figure 1, all circles). Each eDNA sample was placed on ice. To control for potential contamination, we carried as a sampling blank, a 500 ml sample of DI water in each cooler (n = 5), and these blanks were treated identically to the sampled river water, except that it was not opened at the field sites (Carim et al., 2016). In the laboratory, we filtered the sampling blanks and also inserted extraction blanks controlling for contamination (500 ml of DNA-free DI water prepared in the laboratory, n = 5) as negative controls after filtering the field samples. The eDNA in these controls allowed us to
identify any field, transportation, filter equipment, or background contamination.

2.6 | Sample filtration and extraction

After sampling, upon return to the laboratory, we immediately vacuum-filtered all samples through 1.2-μm cellulose nitrate filters using sterile glass 300 ml filter cups. We stored filters in clean Ziplock bags at -4°C until extraction. To extract the DNA from the filters, we followed standard methods (Doi et al., 2015). Each filter was placed into Salivette tubes (Sarstedt) using clean, bleached forceps. For every eight samples, we included a filter-less blank (i.e. tube with all reagents) to serve as a negative control. We submerged the filters in a solution of 400 μl Buffer AL (Qiagen) and 40 μl Proteinase K (Qiagen), and then incubated the tubes at 56°C for 30 min. We centrifuged these tubes at 5,000 g for 5 min, followed by the addition of 220 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8) onto the filters and again centrifuged at 5,000 g for 5 min. In a DNA extraction room, we isolated eDNA from filters using the DNeasy PowerWater Kit (Qiagen 14900-100-NF) following manufacturer protocols (Deiner, Walser, Mächler, & Altermatt, 2015; Doi et al., 2017; Eichmiller et al., 2016). The final volume of the extracted sample was standardised to 100 μl with Buffer AE of the DNeasy PowerWater Kit, and the samples were stored at -4°C until quantitative polymerase chain reaction (qPCR) assay.

2.7 | Quantitative PCR methods

We assayed all DNA extractions in triplicate with qPCR using the following procedure: 10 μl of 2× SensiFAST probe Master Mix (Bio-Rad), 0.8 μl of each 10 μM forward and reverse primer, 4.0 μl of extracted DNA, and 4.2 μl of sterile water. We document the sequences for the primers, probe, and standard in Table 2 (Amberg & Merkes, 2016). We used the following cycling parameters: a single step at 98°C for 2 min; 50 cycles at 95°C for 10 s, 64°C for 20 s, then 72°C for 30 s. To quantify the DNA copy number in each DNA extract, we determined the copy number of a synthesised standard (gBlock, Integrated DNA Technologies) by dividing the molecular weight by Avogadro’s number. We ran a serial dilution of the standard on each qPCR plate and generated a regression line from which the unknown copy numbers of the DNA extracts could be estimated. In addition to the DNA extracts and standard curve, each qPCR plate included a non-template control with sterilised water as template to monitor for contamination at the qPCR set-up step. We ran all qPCR assays on a LightCycler 480 (Roche) real-time PCR system and analysed with associated software. We found no evidence of zebra mussel eDNA in all pre-experiment, cooler, extraction negative controls and qPCR non-template controls. On each plate, the accepted standard curve efficiency ranged from 95% to 98%, and R² ranged from 0.90 to 0.98. Each sample concentration used for statistical analysis was the mean of triplicate qPCR wells. We detected no positive eDNA in the sequential blanks (field, extraction, laboratory). While the primer and probe were not validated against quagga mussels, we report no visual evidence for quagga mussels at the time of our study.

2.8 | Estimating net riverine eDNA retention

Downstream transport is envisioned as a spiral, representing times between episodes of transport, deposition, and resuspension (Workshop, 1990). Previous studies have validated the eDNA spiralling technique in experimental streams (Jerde et al., 2016; Shogren et al., 2017), but this approach has not been attempted in a large river system, nor in any natural flowing waters as far as we are aware. Therefore, using the natural population distribution of zebra mussels in the Gudenaa River, we calculated net transport distance and eDNA deposition in the area devoid of zebra mussels (Haggard, Storm, & Stanley, 2001) (Figure 1). To calculate average eDNA uptake length (Sw), we plotted the natural log of eDNA concentration against distance downstream, fitting data to the simple relationship that describes stream spiralling: In Nw = ln N0 - kx. In this model, N0 is the concentration of the source (i.e. the mean of the sites upstream of the cutoff point of zebra mussels), Nw are eDNA concentrations taken every x m downstream, and the slope k is the longitudinal loss rate (Workshop, 1990). When the regression is statistically significant (i.e. R² > 0.8, p < 0.05), the inverse of the slope, k, represents the average distance eDNA would move downstream before being deposited, with the metric known as Sw (m) (Jerde et al., 2016; Shogren et al., 2017). Because the Sw metric is strongly influenced by discharge, confounding comparisons among river systems of varying size, we also estimated eDNA depositional velocity (vdep, i.e. how fast an eDNA particle moves towards the streambed) as (discharge/width)/Sw (Haggard, Stanley, & Storm, 2005; Jerde et al., 2016; Shogren et al., 2017). We also compared the measured eDNA Sw metric to a linear model representing degradation only, using previously published eDNA degradation rates for mussels, k = 0.029 ± 0.0093 hr⁻¹ (Sansom & Sassoubre, 2017).

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer (3′-5′)</td>
<td>TGGGACACGTCTTAGTT</td>
</tr>
<tr>
<td>Reverse primer (3′-5′)</td>
<td>CAAGCCCATGAGTGTTGACA</td>
</tr>
<tr>
<td>Probe (3′-5′)</td>
<td>6FAM-CTGCCCTTGGTG</td>
</tr>
<tr>
<td>gBlock standard</td>
<td>TGTGGGGCTGCGCTTGGGGACGGGTTTTAGGTATTTATTCTTCAGAGCTTAAGGGCACCCTGGAAGCGCTCTGGTTGATGTTGCTAGTAATGT GTAATTGTCACCACCATGGGCTTTATAATTGT GTGCTAG</td>
</tr>
</tbody>
</table>

**TABLE 2** Zebra mussel primers (as in Amberg & Merkes, 2016) used in this study.
2.9 Statistical analyses

First, we evaluated the effect of zebra mussel density, sampling location and physiochemical variables on eDNA concentrations, comparing two-way correlations (Figure S1). We used analysis of variance (ANOVA) to test for differences between eDNA concentration (sample collection = the mean of triplicate qPCR) among transects and to further test for differences among sample concentrations at each transect site (side versus centre). We then used generalised linear models (GLMs) to predict eDNA concentrations and zebra mussels, as we had a combination of categorical (e.g. substrate type) and continuous variables (e.g. concentration). Because our dataset included samples and surveys taken longitudinally from the same river reach, they were deemed spatially autocorrelated in a Moran’s I test using R package ape (Paradis & Schliep, 2019). To account for this, we included a weighted spatial autocovariate for eDNA and zebra mussel distributions using the spdep package in R (Bivand, Pebesma, & Gómez-Rubio, 2013) to be used as predictor variables in each respective model (Dormann et al., 2007). The autocovariates were weighted by Euclidian distance, with neighbouring sites weighted more heavily than distant sites. For the entire dataset along the river reach, we first used a GLM with a Gaussian distribution to explore relationships between eDNA concentrations and site characteristics (Full model: log(eDNA concentration) ~ zebra mussel density + transect + site + velocity + macrophyte cover + temp + pH + substrate type + chl a + AFDM + nutrients + interaction terms). Here, we used a Gaussian distribution to predict the continuous, normally distributed eDNA concentration response with the listed parameters. We tested for any significant parameter interactions and, when significant, kept them in the full model. We then used a stepwise model selection procedure to select the model with the lowest Akaike information criterion (AIC) value, which we reported as the reduced model. Then, we evaluated if zebra mussel density can be predicted by eDNA concentrations, by setting zebra mussel density as the response variable. We used another GLM with binomial distribution to test for relationships between zebra mussel density and eDNA using R package glm2 (Marschner, 2011) (Full model: zebra mussel density ~ log(eDNA concentration) + site + velocity + macrophyte cover + temp + pH + substrate type + chl a + AFDM + nutrients). For this model, we used a binomial distribution for the zebra mussel density response, which represented the percent cover as a proportion from 0 to 1. We standardised factors (mean of 0 and standard deviation of 1) using the beta.glm function in R. For both eDNA ~ parameters and zebra mussel ~ parameters models, we selected the final model via a stepwise procedure using AIC. Finally, for a subsection of the data, we fit linear models to determine the effect of biological and physical factors on the eDNA concentration (see section above). We performed all statistical analyses in R (Version 3.5.0) (R Core Team, 2014). For creating the map of sampling locations (i.e. Figure 1), we used ArcMap with a Danish baselayer (source: DIVA-GIS and ESRI).

3 RESULTS

3.1 Hydrologic characteristics of the Gudenaa River

At the first logging sensor (2,900 m), we estimated v as 0.9 m/s, Q as 17,300 L/s, and t as 2.5 hr. At the second sampling station (6,600 m), v was 0.2 m/s, Q was 18,550 L/s, and t was 6.5 hr. We report estimated velocities at each longitudinal transect in Table 1. Water velocities tended to be higher in areas with no observable zebra mussel populations, but this trend was not statistically significant (ANOVA, F_{(df = 85)} = 2.06, p = 0.15).

3.2 Spatial distribution of zebra mussels

We found that longitudinally, zebra mussels were distributed variably throughout the study reach, but preferentially occupied shallower and wider parts of the channel. We found the highest density (75%–100% cover) of zebra mussels in the section between Silkeborg Bridge (0 m) and Resenbro Bridge (Figure 1), with zebra mussels making up the majority of the benthic structure of the streambed. We found low or no visual evidence of zebra mussels as the river became more constrained, with densities between 0 and 25% around Transect 13 (Figure 1). From 6,010 to 6,964 m (Figures 1 and 3), where the channel was too deep for substrate sampling, we again found no visual evidence of zebra mussels.

3.3 Spatial distribution of zebra mussel eDNA

We found positive detections (>0 copies/ml) of zebra mussel eDNA throughout the Gudenaa River (Figures 2 and 3). At each transect, eDNA concentrations were not statistically different among samples taken along the sides and in the centre (ANOVA, F_{(df = 29)}; p = 0.41); in general, samples taken from the centre were higher in concentration than those taken from the side. Longitudinally, we detected the highest eDNA concentrations at sites located c. 1,900–3,200 m downstream (transects 15–21), and the lowest eDNA concentrations (mean < 75 copies/ml) in the intervals 0–163 m, 668-1,985 m, and 3,532–6,010 m (ANOVA_{(df = 85)}; p < 0.05; Figure 3).

We further assessed relationships between eDNA concentration and density of the mussels on the stream bottom using stepwise selection GLMs. While the full model included all measured parameters (Table 3, A. eDNA ~ parameters), the parameters selected in the stepwise procedure that best predicted eDNA concentration with reduced AIC included water column ammonia (p = 0.003) and phosphate (p = 0.04) concentrations, zebra mussel cover (p = 0.5), chl a (p = 0.07), and water velocity (p = 0.012). The sampling location along each transect (i.e. side versus centre) did not have a significant effect on the variation in observed eDNA concentration (p = 0.06, Table 3). Even though we found no effect of sampling location on eDNA concentration, we found evidence that the variation within each transect (as coefficient of variation, CV %) changed predictably depending on the proximity...
to areas densely covered with zebra mussels. The within-transect CV (based on the mean of three samples) was lowest in areas in close proximity to dense zebra mussel populations and was higher with increasing distance from dense zebra mussel bed (Pearson’s correlation $\text{df} = 29; R = 0.24, p = 0.03$).

### 3.4 Relationships between zebra mussel density and eDNA concentration

First, we used a simple linear model to explore if zebra mussel density could be predicted by measured eDNA concentrations. While we found a significant relationship between the two factors, the relationship was not strong (linear model, $R^2 = 0.02 p < 0.01$, Table 3). We consequently explored the relationship between zebra mussel density, biophysical parameters, and eDNA concentration further with another GLM, with zebra mussel density as the response variable (zebra mussels ~ parameters). When we performed the stepwise selection procedure, only nitrate ($p = 0.007$), substrate ($p < 0.001$), and sampling location ($p < 0.001$) were included as variables in the simplest GLM (Table 3), and in the full model, zebra mussel density was not predicted by eDNA concentration ($p = 0.7$).

### 3.5 Estimating eDNA spiralling

The spatial variability of zebra mussel beds created a unique study system where we could use simple models to tease apart the role of two dominant mechanisms thought to remove eDNA from the water column: physical retention and biological degradation. Using the dense zebra mussels as a natural source of eDNA, we examined the eDNA concentration data from transects 19–26 only, to estimate the average distance an eDNA particle is transported along this section of the Gudenaa River, expressed as the uptake length $S_w$. Regressing the natural log of eDNA concentration over distance (linear model: $R^2 = 0.84, p = 0.028$, Figure 4), and using the slope ($k$) of $-0.0009$ m$^{-1}$, we estimated the average eDNA $S_w = 1.052 \pm 74$ m. Moreover, the eDNA depositional velocity ($v_{\text{dep}} = 0.53 \pm 0.01$ mm/s) fell within the previously estimated rates of eDNA deposition (fish eDNA $v_{\text{dep}} = 0.18–0.54$ mm/s; Shogren et al., 2017).

![Figure 2](image-url)
In this study, we took advantage of the natural distribution of a sessile organism in a large river system, quantifying the spatial distribution of zebra mussel eDNA concentrations in relation to the target species density. In theory, the concentration of mussel eDNA in a parcel of water should be controlled by several processes, including production and shedding rates, degradation, as well as other removal mechanisms such as physical retention by the river bottom and associated biology such as biofilms and macrophytes. While previous studies have addressed these mechanisms for eDNA removal in experimental streams (Jerde et al., 2016; Shogren et al., 2017), we demonstrate the relationships, and their complexities, empirically in a natural experiment. Our results, derived from a relatively controlled natural experiment, contribute to the growing field of eDNA ecology (Barnes & Turner, 2016), and highlight critical knowledge gaps that must be overcome to accurately relate eDNA concentrations to estimations of species density or abundance.

First, our results demonstrate the challenges associated with predicting biomass with eDNA concentrations in riverine environments (Buxton, Groombridge, & Griffiths, 2017; Shogren et al., 2017). We found an abrupt transition zone between a densely populated riverbed and no physical evidence of zebra mussels (>75% cover) and grey (<50% cover) circles. Transects without zebra mussels are noted by squares. We used the square data points for estimation of net riverine eDNA retention.

Second, our findings underscore that riverine measurements reflect a complex balance of zebra mussel density, eDNA production, transport, and removal. In our study, the lagged relationship between zebra mussel density and overlying eDNA concentration suggests a downstream accumulation of eDNA as water moved over the densest zebra mussel beds (>75% cover). In this case, higher eDNA production outpaced physical and biological removal processes such as deposition or degradation (i.e. transport > removal). Then, eDNA concentrations declined steadily with downstream distance, probably because removal processes exceeded eDNA production (i.e. removal > transport). This area represented a transition zone where
we detected high eDNA concentrations despite species absence. Downstream of this transition zone, where there were no zebra mussels present, we observed the dominance of removal processes (i.e. removal > production). While our GLMs indicate weak relationships between eDNA concentration and zebra mussel density, the downstream offset between the highest eDNA concentration and the most densely populated streambed is likely to explain the general lack of explanatory power in our models.

In addition to revealing that production, transport, and removal can be important processes controlling riverine eDNA, we used our study system to partition influential removal processes. Retention (i.e. disappearance from the water column) reflects a combination of physical removal and biological degradation. Previous studies have been conducted over shorter timescales (c. 2–4 hr) where degradation did not significantly influence eDNA detection, and thus only measured physical eDNA retention (Jerde et al., 2016; Shogren et al., 2017). However, given longer travel times in the Gudenaa River (e.g. 6.5 hr from top to bottom of 7-km study reach), we modelled estimated degradation relative to total retention to isolate the role of physical retention. Using previously reported values for mussel eDNA degradation (Sansom & Sassoubre, 2017), we fit transport models ($C_x = C_0 e^{-kx}$, where $C_x$ is the concentration at a distance $x$, $C_0$ is the initial concentration, and $k$ is the longitudinal mass loss rate) under three different scenarios: (1) where eDNA behaved conservatively (i.e. eDNA declines only from dilution); (2) eDNA declined only as a result of biological degradation (Sansom & Sassoubre, 2017); and (3) eDNA declined due to both biological degradation and physical retention. With this approach, we were able to estimate the role of physical retention relative to other eDNA removal processes (e.g. degradation), given minimal dilution. The difference between the linear models for Scenarios 2 and 3 isolates the effect of physical retention alone and, based on this simple model over distance, physical retention represented 50–80% of eDNA removal compared to biological degradation.

Despite their potential importance, physical or biological eDNA removal is often not explicitly considered when estimating downstream transport. While eDNA may be transported many kilometres from the source (Deiner & Altermatt, 2014; Jane et al., 2015), the assumption that river networks will conservatively integrate eDNA signals may be an over-simplification in heterogeneous environments (Deiner et al., 2016). For mussels, Sansom and Sassoubre (2017) used experimentally derived eDNA release and degradation rates to develop a one-dimensional mass balance model to estimate how far downstream mussel eDNA might be detected, and found that shedding rates balanced decay, estimating that mussel eDNA could move up to 35 km downstream. However, such an advection-dispersion model does not take into account physical or biological removal processes that can be hydraulically- or biologically-mediated (Shogren et al., 2016, 2017) thus the projected 35-km travel distance resulting from the simplified model is likely to be an overestimation. For example, using a degradation coefficient alone, eDNA in the Gudenaa River could potentially travel up to 19 km before becoming undetectable; however, when retention and degradation are both considered, the distance is reduced to c. 6 km (or 68%). The downstream transport of eDNA and its simultaneous removal via physical retention and biological degradation must be considered if eDNA concentrations are to be used effectively to estimate the location and abundance of target species. For example, to optimise sampling strategy, retention rates measured here and in past eDNA work in flowing waters could be used to constrain distances between sampling points in the field. For example, in rivers similar to the Gudenaa River, we would recommend <1,000 m between sampling points, lest eDNA removal processes mask the presence of target species within a river reach.

A major advantage of the eDNA approach relative to traditional sampling methods is the ease of execution at the watershed scale, but to extend its application to estimate density using eDNA concentration data in flowing waters relies in part on the assumption that eDNA production rates are related to the abundance of
the target species. Further, other fundamental challenges must be overcome before informative relative density data can be generated for target species in flowing waters. One challenge of eDNA monitoring is to develop effective molecular primers and probes that are target-specific within the given ecological community. The primer and probe sequences used here were developed for a previous study by Amberg and Merkes (2016) for zebra mussel eDNA detection. They were validated against 27 bivalve and fish species, most of which are known to coexist within the Gudenaa River. The primer and probe were not validated against the close relative of the zebra mussel, the quagga mussel (Dreissena bugensis); however, in our extensive field sampling, we found no visual evidence for quagga mussels in this stretch of the Gudenaa River at the time of our study. We believe our methodology here is generalisable to other study systems, and we encourage potential end-users of the eDNA approach to consider the environmental context of their system, including the local aquatic community and conditions, before adopting the primer and probe sequences used here. The approach taken with this study is a first step towards eDNA application under realistic field conditions, going beyond the foundations provided from more controlled experimental studies. However, these results represent a level of constraint that can be achieved in most field conditions (Jerde et al., 2011) and further emphasises the challenges associated with translating biomass information from eDNA concentrations in flowing waters (Wilcox et al., 2016). The Gudenaa River is hydrologically well-constrained, allowing for a clear demarcation between abundant and absent zebra mussel populations. Additionally, we used a sessile population as our natural eDNA source, while the majority of previous eDNA studies have focused on mobile species such as fish and amphibians (Rees et al., 2014). The role of behaviour, phenology, and species mobility deserve further study in the field of eDNA ecology. Indeed, while our results suggest that it will be challenging to simply back calculate species density or location using eDNA concentration alone, without additional knowledge of the hydrologic conditions of the streambed and water column (e.g., solute concentrations), overlying water velocities, and biological degradation rates, we found clear spatial patterns in the production, transport, and removal of eDNA.

The number of published studies examining eDNA in flowing waters has increased rapidly, and now represents a significant proportion of eDNA-related publications (c. 32%, Roussel et al., 2015), yet the effects of downstream transport and removal mechanisms that strongly influence any measured eDNA concentrations have rarely been addressed. While it remains an open challenge, accurate prediction of species density will require further information about environmental variables, including benthic structure, and hydraulic behaviour, due to the complexities of potential mechanisms driving eDNA transport and retention along a heterogeneous stream or river reach. These results also emphasise the complexity of interpreting the relationship between eDNA concentration and species density. Improved use of eDNA tools will depend on the interface of further work on the ecology of eDNA (Barnes & Turner, 2016) and further hydrological modelling that will disentangle the processes that control eDNA movement in aquatic systems.

ACKNOWLEDGMENTS

We thank A. Brandstrup, B. Tagesen, O. Zahrtmann, and T. Søgaard for their generous assistance with sample collection and processing. This publication was developed under A.J.S.’s STAR Fellowship Assistance Agreement no. FP-91781601-0 awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by the EPA. S.P.E., J.L.T., and D.B. were supported by USDA Grant 2013-33522-21007. We also gratefully acknowledge the two anonymous reviewers and associate editor, whose comments have greatly improved this manuscript.

AUTHOR CONTRIBUTIONS

A.J.S., T.R., and J.L.T. conceived the study; A.J.S. and T.R. performed all riverine field work; and A.J.S. completed sample processing and analysis. A.J.S., S.P.E., and D.B. assisted with data interpretation and modelling. All co-authors contributed to final data analysis and manuscript writing.

DATA ACCESSIBILITY

The data used for this manuscript will be uploaded to Dryad.

ORCID

Ariel J. Shogren https://orcid.org/0000-0002-1284-3836
Jennifer L. Tank https://orcid.org/0000-0001-9005-9548
Scott P. Egan https://orcid.org/0000-0001-5208-1875
Diogo Bolster https://orcid.org/0000-0003-3960-4090
Tenna Riis https://orcid.org/0000-0003-2501-4444

REFERENCES


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