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ORIGINAL ARTICLE

# Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration

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## Abstract

Environmental DNA (eDNA) can be used to detect the presence and abundance of aquatic organisms from water samples. Before implementing this methodology as a tool for monitoring, more knowledge is needed on variation in eDNA concentrations in relation to species abundance and potential confounding factors. Shedding and decay of eDNA may vary extensively over the season and are dependent on environmental factors such as water temperature and on biological processes such as activity level and reproduction. In lotic systems, eDNA concentrations are also affected by downstream transport of eDNA. Sessile freshwater mussels provide an ideal study system for investigating the relationship between species spatial distribution and eDNA concentrations in lotic systems. We quantified freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentrations at four localities in a natural river with detailed knowledge of mussel spatial distribution: (a) upstream of the known species distribution, just downstream (b) a small and (c) a large aggregation and (d) 1,700 m downstream of the large aggregation. To study seasonal variation, we quantified eDNA concentrations during three periods: (a) in late spring, with cold water and relatively inactive mussels; (b) in mid-summer, with higher water temperature and active mussel filtration; and (c) in late summer, during the release of larvae. Species detection was highly reliable, with no detection of eDNA upstream of the species distribution and complete detection downstream of the large aggregation. Detection success of the small aggregation was low, with 13% of the samples testing positive. Downstream transport was efficient, with no significant decrease in eDNA concentrations over 1,700 m river distance. Seasonal variation was strong, with a 20-fold increase in eDNA concentrations from late spring to late summer, during reproduction. Our results highlight both the potential and challenges of eDNA monitoring in lotic systems.

KEYWORDS

conservation genetics, environmental DNA, freshwater mussels, species detection, unionidae

## 1 | INTRODUCTION

For a fast-growing range of species, environmental DNA (eDNA) sampling has been successfully used to infer species presence from water samples (Kelly et al., 2014). This method has mainly been applied to detect rare and threatened species, invasive species and species that are difficult to monitor with conventional methods (Bohmann et al., 2014; Jones, 2013). Aquatic organisms can be detected using eDNA methods, often at higher sensitivity than with conventional methods (Wilcox et al., 2016). Efforts have also been made to use eDNA concentrations to estimate species abundance (Tillotson et al., 2018). Abundance estimates are challenging due to high variability in both the rates at which DNA is shed into the environment (Sansom & Sassoubre, 2017) and how quickly eDNA degrades (Barnes et al., 2014) and is deposited (Jerde et al., 2016). Such variability may occur among species, but is also dependent on environmental factors such as water temperature, UV radiation and stream bottom substrate (Jerde et al., 2016; Strickler, Fremier, & Goldberg, 2015). Extensive empirical and theoretical work is needed before eDNA-based abundance estimates can be reliably used for monitoring.

The unidirectional water flow in lotic environments creates specific opportunities and challenges for estimating species presence and abundance from eDNA (Shogren et al., 2017). While eDNA degradation and deposition may be rapid (Barnes et al., 2014; Dejean et al., 2011; Jerde et al., 2016; Pilliod, Goldberg, Arkle, & Waits, 2014; Tillotson et al., 2018), downstream transport can be highly efficient (Deiner & Altermatt, 2014; Jane et al., 2015; Sansom & Sassoubre, 2017; Wilcox et al., 2016). Water samples may therefore hold detectable eDNA that has been shed by individuals located considerable distances upstream (Deiner, Fronhofer, Machler, Walser, & Altermatt, 2016; Shogren et al., 2017). Downstream transport of eDNA does at the same time hamper analysis of local presence and abundance of target species because any sample may hold a mixture of eDNA shed both locally and further upstream (Shogren et al., 2017). Knowledge on how environmental variables affect eDNA decay and transport may help to predict eDNA concentrations in dependence of species abundance (Carraro, Hartikainen, Jokela, Bertuzzo, & Rinaldo, 2018; Chambert, Pilliod, Goldberg, Doi, & Takahara, 2018; Shogren et al., 2017; Wilcox et al., 2016). However, only a few field studies have explored the relationship between the spatial distributions of individuals and eDNA concentrations in natural lotic systems (Doi et al., 2017; Spear, Groves, Williams, & Waits, 2015; Tillotson et al., 2018; Wilcox et al., 2016). While some of the existing studies concluded that eDNA concentrations primarily reflect local abundance (Doi et al., 2017; Tillotson et al., 2018; Wilcox et al., 2016), Spear et al. (2015) found extensive downstream transport and accumulation of eDNA.

Freshwater mussels are among the most threatened taxa worldwide and knowledge of their distribution and abundance is crucial for their conservation (Lopes-Lima et al., 2017). Freshwater pearl mussels (FPM, *Margaritifera margaritifera*) were historically distributed across large parts of Europe but have decreased dramatically and are now listed as endangered in the IUCN red list (IUCN, 2017; Lopes-Lima et al., 2017). Freshwater pearl mussels are subject to substantial conservation effort (Geist, 2010) and monitoring programs across Europe (Boon et al., 2019). The species has been studied for its genetic population structure across Europe (Geist & Kuehn, 2005; Geist, Söderberg, Karlberg, & Kuehn, 2010; Stoeckle et al., 2017) which in Northern Europe is linked to host preference (Geist et al., 2018; Karlsson, Larsen, & Hindar, 2014). Conventional monitoring of freshwater mussels is well established for many species, but dependent on expert competence and is typically labour and cost intensive. Visual searches for mussels can be hampered when adult individuals are partly or fully buried in the substratum or when the visibility is poor. Environmental DNA is a highly promising tool for monitoring freshwater mussels and previous studies revealed that freshwater mussels shed DNA that can be detected in water samples (Carlsson et al., 2017; Currier, Morris, Wilson, & Freeland, 2018; Deiner & Altermatt, 2014; Dysthe et al., 2018; Sansom & Sassoubre, 2017; Stoeckle, Kuehn, & Geist, 2016). However, empirical work needs to reveal how eDNA concentrations are affected by stream characteristics such as downstream transport and by seasonal variation. Environmental DNA shedding rates are expected to be high in the reproductive season, as found in other taxa (Buxton, Groombridge, Zakaria, & Griffiths, 2017; Spear et al., 2015), but may also be affected by general activity levels. Seasonal variation in environmental factors may in addition affect concentrations of detectable eDNA via decay and deposition rates. For example, water temperature may negatively affect eDNA concentrations due to faster decay in warmer waters (Strickler et al., 2015). Consequently, knowledge of seasonal variation in eDNA concentration is needed for a correct interpretation of eDNA measurements, and to ensure that water samples are collected at the optimal time of the year, if detection and/or quantification of abundance is desired.

In this study, we aimed to test whether FPM eDNA concentrations primarily reflect local mussel abundance or are strongly affected by downstream transport of eDNA; and whether seasonal variation affects concentrations and transport of eDNA. The sedentary lifestyle of freshwater mussels makes them highly suitable for exploring how eDNA concentrations are affected by species distribution in lotic systems. The small river Draktselva in Trøndelag county, Norway, is an excellent river ecosystem for studying how eDNA from FPM is distributed in time and space. The distribution of FPM in River Drakstelva is well documented from conventional methods. Because the distribution is patchy, large contrasts in the signal of eDNA collected at different locations are expected, which makes River Draktselva suitable for studying downstream transport of eDNA. We collected water samples (a) upstream of the known distribution of FPM, (b) closely downstream of a small aggregation at the upstream limit of the species' distribution, (c) closely downstream of a large aggregation and (d) 1,700 m along-river distance downstream of the large aggregation. We predicted higher eDNA concentrations closely downstream of the large aggregation than

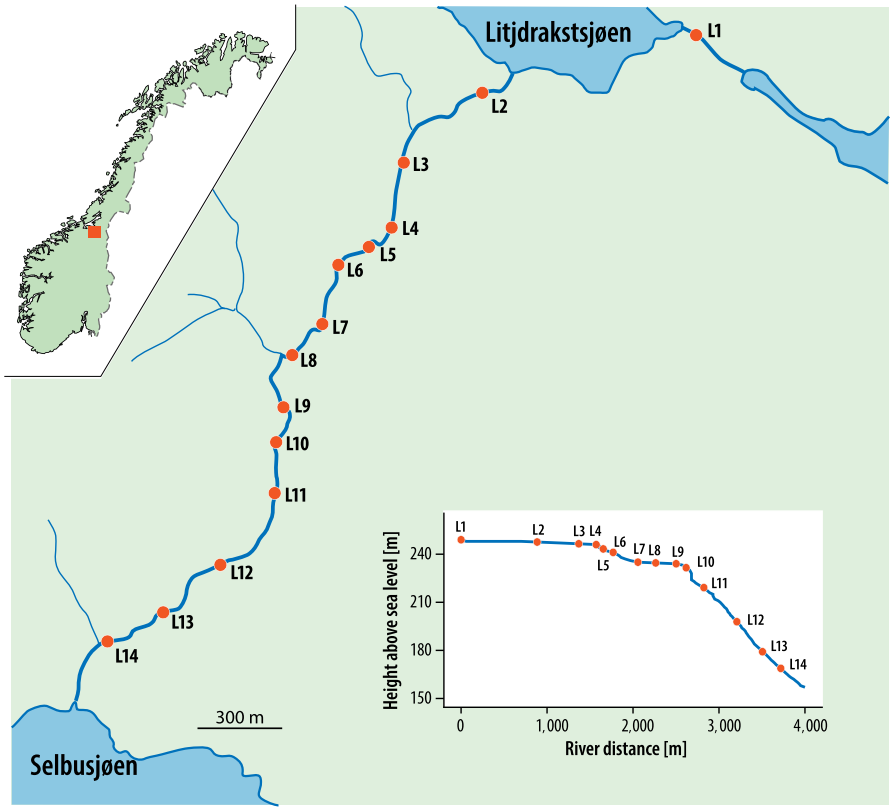


**FIGURE 1** Map and profile of River Drakstelva with sampling localities for eDNA (L1, L2, L7, L14) and conventional recordings of freshwater pearl mussel (L1-L14). The area shown in detail is marked red on the map showing Norway

closely downstream of the small aggregation. We also predicted efficient downstream transport of eDNA and therefore no strong decrease in eDNA concentrations from closely downstream of the large aggregation to 1,700 m further downstream. The locality upstream of the species' distribution served as a negative control, where we expected no detection of FPM eDNA. To explore seasonal variation in eDNA concentrations, we collected water samples during three different times between May and August. Sampling in August was timed to take place when FPM in River Drakstelva is expected to release larvae. Due to increased water temperatures and increased mussel activity in summer, we predicted that we would observe increasing eDNA concentrations during the course of the study.

## 2 | METHODS

### 2.1 | Study river

River Drakstelva (Trøndelag county, Norway) has a forest-dominated drainage of approximately 34.6 km$^2$. The river has a length of ca. 3.5 km, running from Lake Litjdrakstsjøen (248.1 m above sea level) to Lake Selbusjøen (157.1 m above sea level) (Figure 1), with the steepest gradient located in the lower part of the river (Figure 1). At large parts, River Drakstelva is 5 to 10 m wide and shaded by dense vegetation. Discharge is regulated by a hydropower dam further upstream in the river system. Discharge was not recorded but was relatively constant during field work (pers. observation), due to regulations on minimum-discharge (100 L/s) and restricted discharge during summer and autumn. Velocity was not recorded but may be estimated from discharge and river width and depth. Given an average width of 7.5 m and a depth between 0.3 and 0.5 m between localities L7 and L14, velocity under minimum-discharge is between ca. 100 and 160 m/hr. This translates into a maximum travel time of 8 to 14 hr from locality L7 to L14. Discharge was however most likely above minimum when the present study was carried out, suggesting considerably shorter travel times. Small creeks discharging into River Drakstelva do not host FPM and are unlikely to have contributed significantly to total discharge. Water temperature was 4.1°C in May and varied between 13.1°C (locality L2) and 15.3°C (locality L7) in June and between 13.3°C (locality L2) and 13.6°C (locality L14) in August. We also sampled one locality in River Sagelva (locality L1), which is located upstream of Lake Litjdrakstsjøen and upstream of the known distribution of FPM (Figure 1).

### 2.2 | Conventional surveys

Conventional surveys of FPM abundance took place at 14 localities (Figure 1) between 12 July and 24 August 2016. The choice of localities was random with regard to mussel density but aimed to cover all sections of the river and was also affected by practical considerations such as accessibility. Two researchers with extensive experience in monitoring FPM carried out sampling with established protocols (CEN, 2017). At all localities, FPM abundance was recorded in free counts of fixed duration, and at eight localities, abundance was in addition recorded in transects (see Figure S1 for methods). Results from the two methods are well correlated (previous work: unpubl. data; this study: Figure S1) and only free counts

are reported in the Results. In free counts, mussels were directly observed with the help of an aquascope and the number of live individuals encountered during 15 min search recorded. This method is superior over transects in detecting the presence of mussels when densities are low and is therefore used as an additional method for transects in the Norwegian monitoring programme (Larsen, 2017). The person who performs the counts crosses the river from side to side while the number of mussels observed is counted within 15 min duration. At each locality, one search was carried out from fixed starting points in upstream and downstream direction respectively. At River Sagelva (locality L1), conventional surveys were carried out at one locality (three free counts). Conventional surveys did not detect mussels buried in the substratum. Juvenile mussels remain buried in the substratum until they reach a length of ca. 40 mm in River Drakstelva (Larsen, 2017). We have no indication that the relative abundance of the buried mussels differed among localities or that buried mussels occurred were exposed (adult) mussels were absent.

## 2.3 | Water sampling and filtration

Water sampling took place on 3 May, 23 June and 29 August 2017. The main aim of this study was to assess spatio-temporal variation in eDNA concentrations for FPM and we used 0.45 µm sterile filter funnels (Pall MicroFunnel 300 ST) at all sampling localities and times for this purpose. In addition, we tested four other filter pore sizes ranging from 0.22 µm to 2.0 µm during the course of the study (0.22 µm Sterivex-GP Sterile Ventile Filter unit; 0.8 µm Sartorius Cellulose Nitrate Filter; 1.2 µm Sartorius Cellulose Nitrate Filter; and 2.0 µm Merck Millipore glass fibre filter). Testing the effect of filter pore size on eDNA concentrations was not a major goal of this study, but we included results from all filter types in our analyses to maximize sample size and report the effect of filter size. Filter pore sizes used at each sampling locality and season are reported in Table S1.

At each locality, and for each filter type, we collected three parallel water samples, representing the left side, middle and right side of the river. Water samples were collected from the river in bleached 1 L or 10 L plastic bottles and filtrated using a vacuum pump (Microsart e.jet, Sartorius GmbH) connected to a 3-place filter funnel manifold (Pall Corporation) for all filters except the Sterivex syringe filters (Sterivex-GP Sterile Ventile Filter unit, 0.22 µm). For the Sterivex filters, water was manually pushed through the filter using a sterile 50 ml disposable syringe. For the 0.45 µm and 0.8 µm filters, 1 L of water was filtrated; for the 1.2 µm filters, 1.2–2.5 L of water was filtrated and for the 2.0 µm filters 10 L of water was filtrated (Table S1).

After filtration, the 0.45 µm, 0.8 µm and 1.2 µm filters were immediately placed in 2 ml plastic tubes with 1,440 µl ATL-buffer (Qiagen, Hilden, Germany), whereas the 2.0 µm glass fibre filters were placed in 5 ml plastic tubes with 4,050 µl ATL-buffer. For the Sterivex filters, 1,800 µl ATL-buffer was added to the filter capsule and closed in both ends with Luer lock caps. All filters were stored in room temperature until further processing in the genetic laboratory at NINA.

## 2.4 | DNA extraction and genetic analysis

DNA extraction was carried out in dedicated spaces for isolation. PCR-setup was carried out in UV-benches and all work related to PCR and post-PCR-products was carried out in different rooms. Pipettes were sterilized under UV-lamps every day. All filters were extracted using a modified DNeasy Blood & Tissue kit (Qiagen) protocol (Spens et al., 2017). First, proteinase-K (Qiagen) was added to the sample tubes and left overnight at 56°C. For all filters stored in 2 ml tubes, 160 µl proteinase-K was added. For the glass fibre filters stored in 5 ml tubes, 450 µl proteinase-K was added. For the Sterivex filters, 200 µl proteinase-K was added to the capsules. The following day, lysates were transferred to a new tube and AL-buffer and 98% EtOH were added at the same volume as the lysate. The tubes were vortexed and a maximum of 600 µl was sequentially added to a DNeasy spin column and centrifuged. This step was repeated until the entire sample volume had been loaded on to the column. DNA was eluted from the column by adding 100 µl AE-buffer (Qiagen) that had been preheated to 56°C to increase DNA yield, followed by 10 min incubation at room temperature before centrifugation. The DNA-eluate was re-eluted into the same microcentrifuge and incubated for 10 more minutes before a final centrifugation step. The DNA-eluates were kept frozen at −20°C until further analyses.

As a control for the success in detecting present eDNA at all localities and sampling months, water samples were also analysed for brown trout (*Salmo trutta*) eDNA. Brown trout was well suited as a control because it occurs at all parts of the river, including sections were FPM is absent or occurs at low density. Species-specific primers for FPM (Carlsson et al., 2017) and brown trout (Gustavson et al., 2015) were multiplexed in a droplet-digital-PCR (ddPCR) (Bio-rad Laboratories, Inc), using a 6-FAM labelled and a VIC-labelled TaqMan MGB-probe. Both primer pairs target regions within the mitochondrial cytochrome oxidase I (COI) gene and amplify fragments of 83 bp and 61 bp, respectively. Two PCR replicates were run for most of the samples (Table S1).

In a total reaction volume of 22 µl, ddPCR-reactions contained 3.6 µM forward and reverse primers, 0.86 µM of the two probes, $dH_2O$, ddPCR™ Supermix for Probes (No dUTP) (Bio-rad Laboratories, Inc.), and 5 µl or 1 µl DNA template. Samples collected in May were only analysed with 5 µl DNA-template. Samples collected in June were first analysed with 5 µl DNA-template, but the increased eDNA concentrations in June made the segregation between positive and negatives droplets blurry and these samples were therefore re-amplified from 1 µl DNA template. Samples collected in August were only analysed with 1 µl DNA template. In June, the ddPCR run with 5 µl DNA produced ca. 50% higher eDNA concentrations than the PCR run with 1 µl DNA, even after controlling for eluate volume. While it is unknown whether template volumes affected our results, it would render our analysis conservative, because May samples were analysed with 5 µl template DNA and August samples were analysed with 1 µl template DNA. As a negative control, $dH_2O$ was added as template, and as a positive control, DNA extracted from FPM and brown trout tissue was included in each ddPCR run.

PCR droplets were generated in an AutoDG™ Instrument (Bio-rad Laboratories, Inc.). PCR amplification was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems), using the following thermal cycling conditions: An initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 60°C for 1 min, a final step of denaturation at 98°C for 10 min, and a final hold at 4°C. PCR plates were transferred to a QX200™ Droplet Reader (Bio-rad Laboratories, Inc.) for automatic detection of fluorescent signal in the droplets. The QuantaSoft software v.1.7.4 (Bio-rad) was used to separate positive from negative droplets according to the manufacturer's instructions. One of two PCR runs of samples from one locality, month and filter type (locality 11, June, 2 µm pore size) were excluded from statistical analysis because the fluorescent signal allowed no unambiguous separation between positive and negative droplets (Table S1).

In our analysis of eDNA concentration, we treated samples with less than three positive droplets as zeros. This was done to avoid false positives, based on previous experience from eDNA analysis with similar ddPCR protocols (F. Fossøy, unpubl. data). Low frequencies of negative controls with one positive droplet and none with two positive droplets in this study suggest that the chosen threshold was suitable for our analysis of FPM eDNA (see Results).

The target DNA concentration based on the ddCPR-analysis was calculated as:

1. $DNA_{conc}$ = −log(number of negative droplets/total number of droplets)/drop volume

using a drop volume of 0.00085 µl. A standardized measure of DNA copies per litre of water was then calculated as:

2. $DNA_{copies/L} = DNA_{conc} \times$ PCR-volume/Template volume × Eluate volume/ Water volume

where PCR volume was 22 µl, Template volume was 1 or 5 µl and Eluate volume was 100 µl. Water volume varied between 1 and 10 L (Table S1).

## 2.5 | Environmental DNA controls

Freshwater pearl mussel or brown trout eDNA was not detected in any of the field or lab negative controls. Lab negative controls consisted of one to eight samples with $dH_2O$ as template in each of five PCR plates (20 samples total). Each plate also included one positive control (DNA isolated from tissue samples) for brown trout and FPM each. Positive controls for brown trout tested negative for FPM eDNA and vice versa. Positive controls tested positive for the respective target species. A single positive control for brown trout tested negative, but many water samples in the same PCR run tested positive for brown trout eDNA. Field negative controls consisted of samples collected in River Sagelva, above the species' known distribution (locality L1), as well as three samples of $dH_2O$ filtered in the field with 0.45 µm filters (locality L7; two in June, one in August) and one sample of water collected in a creek running into River Drakstelva that is known to not inhabit FPM (see Methods). Field negative controls were collected in River Sagelva instead of River Drakstelva because FPM reached all the way to the outlet of Lake Litjdraktssjøen (Figure 2). River Sagelva is part of the same river system and we have no indication for differences in environmental conditions that could affect the detection of eDNA. Freshwater pearl mussel eDNA was not detected in any of these negative field controls.

## 2.6 | Statistical analysis

We used a linear mixed model (LMM) to test whether FPM eDNA concentrations were affected by sampling locality (Figure 1), sampling month (May, June and August) and filter pore size (0.22, 0.45, 0.8, 1.2 and 2.0 µm pore size). There was a clear-cut difference in eDNA concentration between the two upper (L1 and L2) and the two lower (L7 and L14) localities and we did not perform statistical tests on the difference among those groups. The LMM on FPM eDNA concentration was limited to the two lower localities (L7 and L14) and locality, month and filter were included as fixed factors, and water sample as a random factor. Graphical inspection did not suggest an interaction effect between the fixed factors, and the model was fitted without interaction terms. The locality term in the model tested whether eDNA concentrations differed between L7 and L14 and thus to what extend detectable eDNA was transported downstream. The sampling month term tested seasonal variation in eDNA concentrations. Environmental DNA concentrations were log transformed prior to analysis and model assumptions of normality and homogeneity of variance of residuals validated by graphical inspection. For inference testing, we tested the fit of the full model against models from which the fixed factor of interest was removed. We used *F*-tests with Kenward-Roger approximation (*KRmodcomp* function in the *pbkrtest* R package) because of unbalanced sample sizes (Halekoh & Hojsgaard, 2014). We report inference test statistics for each fixed factor (locality, month and filter) in the text and model estimates and confidence intervals per locality and month in Figure 2. For filter size, model estimates are reported in the text.

In order to maximize sample size and statistical power, we included filters of all pore sizes in our main analysis on the effect of locality and sampling month. Because only 0.45 µm filters were used in all instances (see Water sampling and filtration), we also fitted models restricting data to those samples only and compared results with our main analysis. The restricted models provided similar estimates for the effects of locality and month as models including all filters and results are therefore not reported.

# 3 | RESULTS

## 3.1 | Conventional surveys

Conventional surveys at 13 localities in River Drakstelva revealed a large FPM aggregation in the mid-section of the river. High densities were recorded at localities L5 to L7 and significant FPM densities

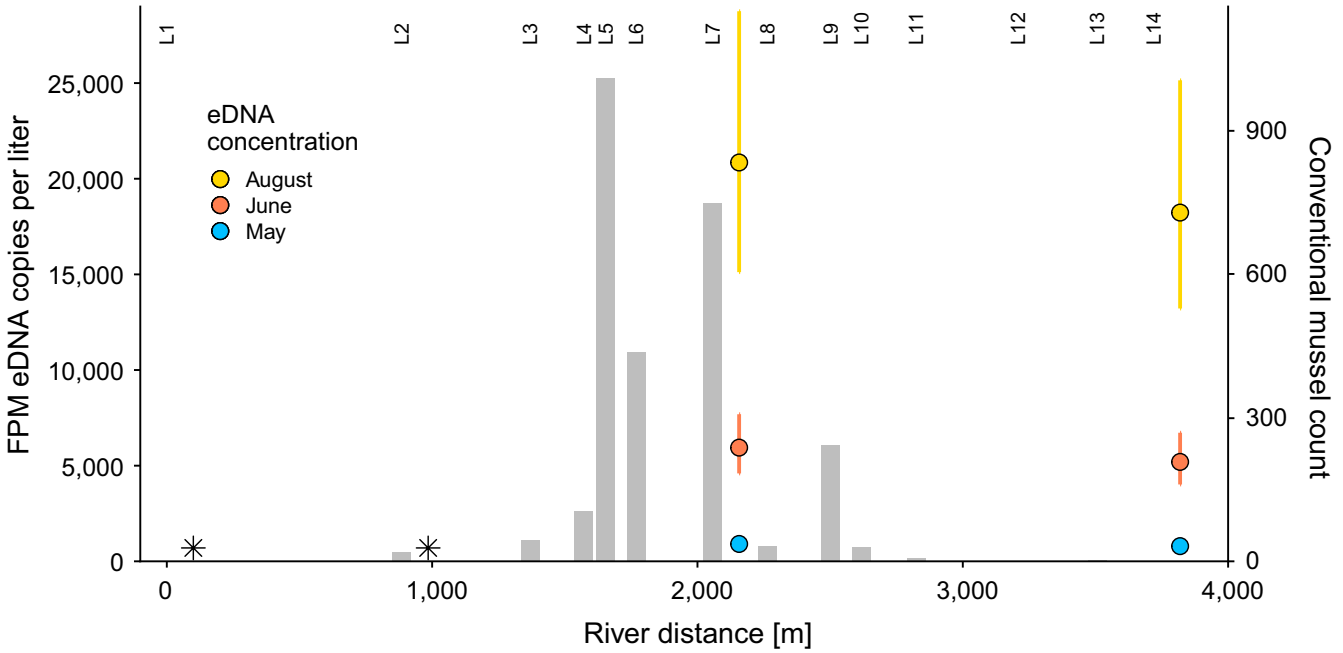


**FIGURE 2** Freshwater pearl mussel (FPM) eDNA concentrations at four localities (L1, L2, L7 and L14) and conventional recordings along river distance of the River Drakstelva water course (distance in metres downstream). Grey bars indicate the number of adult mussels detected during 30 min (2 × 15 min) free search (conventional surveys) at 14 sampling localities (L1-L14). Circles indicate model estimates for eDNA concentrations (copies per litre water sampled) at three times of the season (blue = May, red = June and yellow = August) and error bars indicate 95% confidence intervals. At localities L1 and L2, all and almost all samples tested negative for FPM eDNA respectively, and those localities were not included in the model (marked as asterisk)

were also recorded at locality L9 (Figure 2). No FPM was found at the most downstream locality (locality L14) and only few mussels (range = 1–29 mussels per site, total = 38 mussels) were recorded at the nearest upstream localities (localities L10 to L13) (Figure 2). The distance from locality L14 to the closest larger FPM aggregation (locality L9) was ca. 1,200 m (Figure 2). At locality L10, 29 mussels were recorded within the sampled area (Figure 2) and given the area of suitable habitat, the total number of mussels between L9 and L11 can be estimated to be ca. 100 individuals. A search along the entire river section between that area and L14 revealed very low-mussel densities, which are reflected in mussel counts at L11 to L14 (Figure 2), and which suggest a total of ca. 50 individuals. Mussel densities at the most upstream locality in River Drakstelva (locality L2) were low, with 19 individuals detected during 30 min free search (Figure 2) and 76 individuals detected in transects (Figure 2). Free counts and transects together (95 mussels detected) almost completely covered the river section between the eDNA sampling locality at locality L2 and Lake Litjdrakstsjøen (Figure 2). Conventional surveys in River Sagelva (locality L1) revealed no FPM (Figure 2).

## 3.2 | Environmental DNA detection

A total of 90 filters were analysed for FPM and trout eDNA (Table S1). Freshwater pearl mussel eDNA was detected in all 48 samples collected downstream of the large mussel aggregation (localities L7 and L14) and in all PCR replicates of those samples (78 PCRs) (Figure 2; Figure S2). In contrast, FPM eDNA was not detected in any of the 21 samples (36 PCRs) collected in Sagelva, upstream of the known distribution of species (locality L1). In collections immediately downstream of the smaller FPM aggregation at the upstream limit of the species' distribution (locality L2), FPM eDNA was detected in only three of the 21 samples (all in June, one sample for 0.45, 0.8 and 1.2 µm filter each). For each of those three samples, only one of the two PCR replicates was positive and eDNA concentrations were low (130–251 eDNA copies per litre).

Brown trout eDNA was detected at all sampling localities and at each sampling month. This reveals that lack of detection and low-detection rates of FPM eDNA at localities L1 and L2 respectively were not caused by methodological or environmental factors hampering the detection of eDNA in those water samples. Out of a total of 90 samples, only three samples tested negative for brown trout eDNA (in all PCR replicates) and 10 samples tested negative in one out of two PCR replicates.

## 3.3 | Environmental DNA transport

Despite the absence of FPM at the most downstream locality (locality L14), and only few individuals being found within 1,200 m along-river distance upstream, eDNA concentrations were high and did not differ significantly from samples collected immediately downstream of the large mussel aggregation (locality L7) (LMM: $F = 1.73$; $p = 0.20$; Figure 2). Environmental DNA concentrations were similar between localities L7 and L14 at all three sampling periods (Figure 2 and Figure S2), despite large seasonal differences in

eDNA concentration, indicating an effective transport of eDNA in the 1,700 m stretch from locality L7 to locality L14.

### 3.4 | Seasonal variation

Freshwater pearl mussel eDNA concentrations varied much over the season (LMM: $F = 176$; $p < 0.001$; Figure 2 and Figure S2). Concentrations at localities L7 and L14 increased more than 20-fold from under 1,000 eDNA copies per litre in May to about 20,000 eDNA copies per litre in August, when FPM are expected to release their larvae. A heterogenous distribution of larvae in the water may have resulted in strong variation in eDNA concentrations in August, but variation among water samples was low (Figure S2). High FPM eDNA concentrations in August were not reflected in an increased eDNA detection rate downstream of the smaller FPM aggregation (L2), where only samples collected in June tested positive (see above).

### 3.5 | Filter pore size

Freshwater pearl mussel eDNA concentrations were significantly affected by filter pore size (LMM: $F = 10.24$; $p < 0.001$; Figure S2). Filters with 2.0 µm (collected in May and June) and 0.22 µm pore size (collected in August) resulted in lower FPM eDNA concentration than 0.45 µm filters (contrast: 2.0 µm: −0.73 ± 0.14 log eDNA copies per litre; 0.22 µm: −0.74 ± 0.22 log eDNA copies per litre). Filters with 0.8 µm and 1.2 µm pore size (collected in June) did not differ from 0.45 µm filters (contrasts: 0.8 µm: 0.02 ± 0.18 log eDNA copies per litre; 1.2 µm: −0.14 ± 0.18 log eDNA copies per litre).

### 3.6 | Samples below positive threshold

To avoid false positives, we treated samples with less than three positive droplets (out of ca. 6,000–17,000 droplets in each ddPCR) as negatives, that is as eDNA concentration of zero copies per litre. Only 5% of the lab negative controls for FPM ($N = 20$) had one positive droplet and none had two or more positive droplets. In field samples from upstream of the species' distribution (locality L1), where all samples tested negative for FPM eDNA, 11% of the samples ($N = 36$) had one positive droplet and no sample had two or more positive droplets. Downstream of the small FPM aggregation (locality L2), where only a small proportion of samples tested positive for FPM eDNA, 15% of the negative samples ($N = 33$) had one positive droplet and 15% had two positive droplets. The median number of positive droplets for PCRs testing positive for FPM eDNA ($N = 81$) was 60 (range = 3–384 droplets) and only 4% had less than 10 positive droplets. For brown trout, 32% of the lab negative controls ($N = 19$) had one positive droplet and none had two positive droplets.

## 4 | DISCUSSION

Our results show that downstream transport of FPM eDNA can be highly efficient, with no substantial loss of detectable eDNA occurring over ca. 1.7 km river distance. Given our finding that larger FPM aggregations can be detected over long distances, rivers may be efficiently surveyed for such aggregations by collecting water samples in accordingly large intervals. Downstream transport may at the same time hamper monitoring of local abundance, because eDNA concentrations are affected by mussels located both nearby and further upstream, and collection of eDNA with closer distances between localities is needed to identify shorter stretches of high or low FPM densities. With a better understanding of eDNA transport and decay, this may be addressed by models that account for these variables (Carraro et al., 2018; Cerco, Schultz, Noel, Skahill, & Kim, 2018; Sansom & Sassoubre, 2017). The same models may also be used to determine sampling intervals that are needed to reliably detect FPM occurrences of a given size.

Accumulation of eDNA during downstream movement of water across the large mussel aggregation led to high eDNA concentrations at locality L7. Efficient transport of eDNA explains that concentrations were equally high 1,700 m downstream. Smaller amounts of eDNA were likely added by the smaller mussel aggregations located closely downstream of L7 and similar amounts of eDNA were lost during downstream transport. Previous studies on downstream transport of eDNA have reported highly variable results on eDNA detection distances (Deiner & Altermatt, 2014; Pilliod et al., 2014; Pont et al., 2018; Stoeckle et al., 2016) and changes in eDNA concentration during downstream transport (Jane et al., 2015; Nukazawa, Hamasuna, & Suzuki, 2018; Sansom & Sassoubre, 2017; Tillotson et al., 2018; Wilcox et al., 2016). Environmental DNA detection distances varied between less than 50 m (Pilliod et al., 2014) to more than 100 km (Pont et al., 2018). Studies that have quantified eDNA concentrations have found either decreasing concentrations (Nukazawa et al., 2018; Tillotson et al., 2018) or stable concentrations (Sansom & Sassoubre, 2017) over river distances between 250 m and 3 km. In line with our results, Deiner and Altermatt (2014) detected freshwater mussel (*Unio tumidus*) eDNA 9 km downstream of the source and Sansom and Sassoubre (2017) found stable freshwater mussel (*Lampsilis siliquoidea*) eDNA concentrations over 1 km river distance. In contrast, the only previous study addressing downstream transport in FPM reported moderate eDNA detection rates 25 m downstream, but no detection 500 m and 1,000 m downstream of large FPM aggregations (Stoeckle et al., 2016).

Highly variable results on downstream transport are expected from differences in methodology (e.g. detection sensitivity), study species and stream characteristics. Stream characteristics that may affect downstream transport of eDNA include discharge, gradient and stream bottom sediment (Jerde et al., 2016; Strickler et al., 2015). Pont et al. (2018) found that a model of the effect of water depth and velocity on eDNA sedimentation largely explained variable detection distances in empirical studies. In a caged fish experiment, discharge had a strong effect on the decrease of eDNA concentration during downstream transport (Jane et al., 2015). Downstream transport in the present study may have been facilitated by a steep gradient in the studied river section. We did not measure discharge or velocity, but estimated discharge (see Methods) suggested a short eDNA travel time in the magnitude of a few hours. Decay was therefore unlikely

to be a main factor reducing downstream transport. A steep gradient, together with hard stream bottom and turbulent mixing due to larger rocks and cascades, likely also reduced sedimentation of eDNA. This may explain why we found stable eDNA concentrations, while the model by Pont et al. (2018) predicts sedimentation of a large part of eDNA over the studied distance for the stream characteristics (water depth and velocity) of River Drakstelva (Figure S3 in Pont et al. (2018)). Future studies need to show to what extend our results are transferable to other FPM populations and different stream conditions.

The strong seasonal increase in eDNA concentrations was most likely explained by higher DNA shedding rates later in the season, rather than by lower decay rates. Freshwater pearl mussels are filter feeders, and activity drives their shedding rates. When feeding rates are high, more water volume is passing through their body and more faeces are produced, leading to higher shedding rates (Sansom & Sassoubre, 2017). Environmental DNA concentrations were much higher in August, as expected from the release of larvae. Female FPM develop large numbers of eggs that develop into larvae (several millions) that are released into the water and transported to a suitable fish host. Within populations, larvae release typically occurs within a period of 1–4 weeks (Bauer, 1987). Seasonal timing of fertilization and release of larvae varies among populations, but peaks in the studied population at the time water samples were collected in August (Larsen, 2017). High eDNA shedding rates during the reproductive season have previously been observed in amphibians and fish, related to the release of gametes and larvae (Buxton et al., 2017; Doi et al., 2017; Spear et al., 2015). The seasonal increase in eDNA concentrations was probably not caused by decreased decay, as decay would be expected to be faster in warmer waters. Also, downstream transport of detectable eDNA did not differ across the season, suggesting no change in decay and settlement. This is in line with previous studies on seasonal variation in eDNA concentrations, finding that effects of shedding rates are most important, overriding potential seasonal effects of decay rates (Buxton et al., 2017).

Detection success was low for the small mussel aggregation at the upstream limit of the species' distribution. Water samples were collected immediately downstream of the small aggregation, consisting of approximately 100 mussels. Detection success did not improve when larger water volumes were filtered with wider pore size in this study. Only a low proportion of samples tested positive and with only three to five positive ddPCR droplets (out of 10,000–16,000 droplets). This was only marginally above the positive threshold used in this study (minimum three positive droplets) and translated to only 130 to 250 copies eDNA per litre water. Densities at sampling localities upstream of locality L7 suggest that the large mussel aggregation consisted of more than 10,000 mussels (Larsen, 2017) and was thus at least 100 times larger than the small aggregation. Environmental DNA concentrations downstream of the small and large aggregations may be expected to be approximately proportional to the number of mussels located upstream, given our results of efficient downstream transport. Dividing eDNA concentrations measured at the large aggregation by 100 (10,000 mussels vs. 100 mussels), expected concentrations at the small aggregation were at the limit of detection in August (ca. 210 copies per litre) and below the limit of detection in June (ca. 60 copies per litre) and May (ca. 10 copies per litre). While this is a simplification, the expected concentrations fit well with our observed concentrations from eDNA PCR-amplifications. Notably, models on decay and accumulation of eDNA would be needed to predict eDNA concentrations along larger river distances and with more complex distributions of mussels. Previous studies on the detection of freshwater mussel eDNA in natural systems have either targeted much larger mussel aggregations (Stoeckle et al., 2016) or do not report individual counts (Currier et al., 2018; Deiner & Altermatt, 2014; Dysthe et al., 2018; Sansom & Sassoubre, 2017). Carlsson et al. (2017) report reliable detection of an FPM aggregation of 62 individuals, but it is unknown to what extent downstream transport of eDNA may have explained those results. Comparison with other aquatic organisms is limited by for example different eDNA shedding rates, but high-detection rates have been found for very low densities, such as less than one fish per 100 m river (Wilcox et al., 2016). Detection of the small mussel aggregation in our study may have been affected by small-scale effects of sampling location (Carlsson et al., 2017). Water samples were collected only ca. 50 m downstream of the aggregation and all three water samples that tested positive for eDNA were collected at the side of the river at which most of the mussels were located. Future studies need to reveal the river distance at which detection success is highest, given sufficient mixing of eDNA in the water column and minimal loss of eDNA due to decay and settlement.

Our results have important implications for monitoring FPM with eDNA. Together with previous studies (Carlsson et al., 2017; Currier et al., 2018; Sansom & Sassoubre, 2017), our results show that larger freshwater mussel aggregations can be reliably detected with eDNA. At the same time, efficient downstream transport, strong seasonal variation in eDNA concentrations and limits in the detection of small mussel aggregations emphasize that surveys need to be carefully adjusted to the study aims. For example, comparisons of eDNA concentrations or detection rates among localities or populations are only possible when samples are collected at the same time of the season and when mussels are in the same reproductive state. We did not find a larger variation in eDNA concentrations among samples within localities in August, which may be expected with a likely heterogenous distribution of larvae in the water. Larvae release may have been intense at the time samples were collected, which may have resulted in consistently high concentrations of larvae and thus eDNA in water samples. However, a sparse and heterogenous distribution of larvae may dramatically increase variation in eDNA concentrations and differences in the timing of larvae release may introduce variation when comparing localities within or among rivers. Other times of the year may therefore be preferential for studies quantifying or comparing mussel abundance. In lotic systems, surveys need to consider downstream transport, by suitable choice of sampling localities and according interpretation of the results. Downstream transport of freshwater mussel eDNA has recently been modelled on the basis of species-specific shedding and decay rates (Sansom & Sassoubre, 2017). The

model successfully predicted eDNA concentrations downstream of a freshwater mussel aggregation, but further studies in natural systems are needed for the incorporation of a more complex and thus realistic distribution of mussels along the river.

The field and lab methods used to estimate FPM eDNA concentrations produced highly reliable results. None of the negative controls, including samples collected upstream of the species' distribution (locality L1), tested positive for FPM eDNA, while all samples downstream of the larger FPM aggregation tested positive. This indicates that no contamination occurred during sampling and genetic analysis (Wilcox et al., 2016) and that the sensitivity of the protocol allowed detection of larger FPM aggregations with high reliability. Sensitivity was higher than for an earlier eDNA protocol for FPM that targeted another genetic marker (Stoeckle et al., 2016). High detection rates of brown trout eDNA at all localities showed that the absence of FPM eDNA at localities L1 and near absence at locality L2 was not caused by methodological problems. As expected, filter pore size affected eDNA concentrations, and concentrations were lowest when filters of largest pore size (2.0 µm) were used. Filters of 0.45 to 1.2 µm pore size varied however little in eDNA concentrations and opposite to expectations, filters of smallest pore size (0.22 µl) gave lower concentrations than 0.45 µm filters. Our results suggest that the main conclusions of this study would not be affected by the choice of filter pore sizes within the explored range. In conclusion, the analysis of eDNA concentrations with the applied methods provides a highly promising tool for monitoring FPM.

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## AUTHOR CONTRIBUTIONS

FF and SK initiated the study and BML, FF, RS and SK designed the study. BML, RS and SK carried out field sampling and HB conducted laboratory analyses. FF and SW analysed the data and SW drafted the first version of the manuscript. All the authors contributed to revisions.

## DATA ACCESSIBILITY

Data will be archived on Dryad.

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