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RESEARCH ARTICLE

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Dead or alive — Old empty shells do not prompt false-positive results in environmental DNA surveys targeting the freshwater pearl mussel (*Margaritifera margaritifera* L.)

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Abstract

1. Environmental DNA (eDNA) from water samples is increasingly used to detect the presence and distribution of species in aquatic ecosystems. However, before implementing eDNA in monitoring programmes, various species-specific sampling or analytical issues remain to be resolved in order to minimize frequencies of false-positive and -negative results. For example, empty shells from freshwater pearl mussels (*Margaritifera margaritifera*) contain extractable DNA (chemical extraction from ground-up shells) suggesting a risk of false-positive samples at stream sites with extinct populations but with empty shell material remaining.
2. The aim of this study was to investigate whether empty and naturally degrading shells from *M. margaritifera* can cause false-positive eDNA signals in water samples.
3. Water samples were collected from outdoor stream channels (in Lemming, Denmark) with living freshwater pearl mussels or empty shell material (density ~10 individuals m^{-2}) during a 3-week experimental period. Living freshwater pearl mussels were collected from Hemgravs stream in Sweden and transported to Denmark according to permissions granted by the Swedish and Danish authorities.
4. All water samples from stream channels containing empty shells were negative for eDNA indicating that eDNA traces in stream water are most likely to originate from living individuals located upstream of the sampling site. Water samples collected from stream channels containing living individuals of *M. margaritifera* were consistently positive for eDNA except for one sample (interpreted as a false negative).
5. The study shows that positive eDNA signals for freshwater pearl mussels most likely reflect the presence of living individuals. Consequently, we suggest that eDNA should be used to locate remaining population fragments of *M. margaritifera* in deep and turbulent streams, providing a platform for faster and

more efficient decision making when launching investigative and mitigation initiatives.

KEYWORDS

conservation, eDNA, endangered species, EU Habitats Directive, freshwater pearl mussels, invertebrates, streams

1 | INTRODUCTION

Freshwater ecosystems are among the most impaired ecosystems on earth with species extinction rates exceeding those of most other types of ecosystem (Strayer & Dudgeon, 2010), and indicators of biodiversity (extinction rates, populations trends, etc.) are increasingly trending downward. At the global level, freshwater mussels have suffered severe population declines in recent decades and are among the most endangered groups of animals in freshwater ecosystems (Strayer et al., 2004; Bogan, 2008; Haag & Williams, 2014). One example is the freshwater pearl mussel (*Margaritifera margaritifera* L.), considered as one of the most threatened species of freshwater mussels (Geist, 2010; Sousa et al., 2015). For example, by 1990, more than 90% of the European freshwater pearl mussel populations in cool lentic systems of the Holarctic region had disappeared, and this negative trend has probably continued or even accelerated since then (Geist, 2010). Consequently, the freshwater pearl mussel is listed in Annex II and Annex V of the European Habitats Directive ensuring that European Union (EU) member states have legal obligations to protect and improve conditions for the remaining populations (Council of the European Communities, 1992). Annex II requires that member states designate special areas of conservation for species listed, where the special areas of conservation contribute to the European Natura 2000 network. Annex V lists "species of Community interest whose taking in the wild and exploitation may be subject to management measures."

Currently, the main concern regarding freshwater pearl mussels is the lack of juvenile reproduction, which may be caused by impairments to host fish populations (*Salmo trutta* and *Salmo salar*), habitat degradation, and poor water quality (Degerman et al., 2009; Geist, 2010). In fact, most European populations have lacked successful reproduction for 30–50 years, but owing to the long lifespan of freshwater pearl mussels (250+ years: Degerman et al., 2009), reproduction may resume in old individuals if suitable environmental conditions are re-established (Geist, 2010). The obvious first step in diagnosing environmental causes for population declines to locate existing populations as performed by several EU member states (see review by Geist, 2010) and in North America (Gasparini et al., 2020).

The newly developed standard of the European Committee for Standardization for monitoring population distributions of freshwater pearl mussels suggests wading of rivers with aquascopes or diving in deeper or more turbid rivers (British Standards Institution, 2017; Boon et al., 2019). However, diving is not only costly and time consuming, the chance of detecting scarce numbers of individuals in larger rivers with limited visibility can be critically low, especially since juvenile life stages mainly occur buried in the stream bed (Degerman et al., 2009). In such situations or where population sizes are small, environmental DNA (eDNA) can be applied as a promising alternative to circumvent the problems with low detection rates in conventional survey methods (Stoeckle, Kuehn & Geist, 2016; Wacker et al., 2019; Togaki, Doi & Katano, 2020).

Although eDNA methods may still fall short in fulfilling the legal requirements of the EU Habitats Directive (quantifying population size, age distribution, and reproductive potential), it holds great promise for effectively monitoring the presence or absence of rare and endangered species, providing an option for mapping population distributions. The eDNA methods can guide subsequent conventional monitoring efforts targeting the quantification of population size, age distribution, and reproductive potential. Moreover, in deep and turbid rivers where conventional monitoring (e.g. diving) is often highly inefficient, eDNA methods may well serve as the only viable option to detect the presence or absence of freshwater pearl mussel populations. Accordingly, several eDNA detection tools have been developed during recent years (e.g. Carlsson et al., 2017; Currier et al., 2018; Gasparini et al., 2020).

Freshwater pearl mussels excrete DNA with their mucus (Palmer, Styan & Shearman, 2008). However, Geist, Wunderlich & Kuehn (2008) succeeded in retrieving DNA from empty shells with an age of approximately 1 month by physical grinding of shell material followed by chemical extraction, whereas no DNA could be extracted from shells with an age of approximately 3 months. The findings of Geist, Wunderlich & Kuehn (2008) suggest that DNA from empty shells may cause false-positive signals in rivers where shells remain but the mussels have become extinct. For example, Stoeckle, Kuehn & Geist (2016) found eDNA from freshwater pearl mussels in a German river with historical population records, but conventional monitoring efforts did not reveal remaining, living individuals. The authors suggested that one plausible explanation could be DNA secretion from remaining shell material causing false-positive results. Another similar example is the River Varde in Denmark, where a recent survey recorded eDNA in water samples from two sites with historical records of freshwater pearl mussel populations (Andersen & Wiberg-Larsen, 2017), but several conventional monitoring attempts (including diving) have not confirmed the presence of living individuals since 1999. These contradictory results between eDNA and conventional surveys in deep and turbid rivers can be: (i) a consequence of insufficient detection probabilities of conventional

monitoring; (ii) false-positive results from eDNA surveys as a result of DNA release from empty shell material or upstream populations; or (iii) resuspension of sediments where DNA degradation may be significantly slower compared with the water column (Turner, Uy & Everhart, 2015; Sakata et al., 2020).

The main aim of the present study was to examine whether empty shell material from freshwater pearl mussels excretes eDNA in detectable amounts. A controlled common garden experiment was conducted in flow-through stream channels using two treatments (living individuals and empty shell material) and a negative control group. Water samples for eDNA analysis were collected with weekly intervals during three experimental weeks. We expected that the potential eDNA secretion from empty shell material would not produce detectable levels of eDNA in stream channel water, as the DNA present in shell material probably needs to be extracted by grinding followed by chemical extraction (Geist, Wunderlich & Kuehn, 2008).

2 | METHODS

2.1 | Experimental set-up

The experiment was conducted in 12 outdoor stream channels in October/November 2019. The stream channel facility is located close to Lemming, Denmark (56°4′ N, 9°31′ E). The channels were 12 m long, 60 cm wide, and 30 cm deep, and the slope was 5‰ for all channels. In each channel, the substrate was supplied to resemble stream-bed characteristics in Hemgravs stream in central Sweden (62°52′ N, 16°50′ E), which harbours a large and ecologically sustainable population of freshwater pearl mussels. The substrate comprised coarse sand and stones (diameter 30–200 mm) using a 1:1 distribution ratio in terms of relative cover. The substrate depth was approximately 15 cm, enabling living individuals of freshwater pearl mussels to be positioned partly buried in the sediment. The stream channels were continuously supplied with water from a neighbouring stream (Lemming stream) with no known or historical population of freshwater pearl mussel, using a central feeder pump. (Also see Neif et al. (2017) for further details on the stream channel set-up.)

2.2 | Experimental animals and shell material

The Swedish authorities (Länsstyrelsen Västernorrland, Dnr. 623-4738-19) granted permission to sample freshwater pearl mussels in Hemgravs stream (62°52′ N, 16°50′ E). The Danish Environmental Protection Agency granted permission (J.nr. 2019-11-711-01003) to import the collected freshwater pearl mussels for experimental use at the Lemming facility. Living individuals of freshwater pearl mussels were collected in November 2019 by hand while wading, and stored in moist conditions in a cooling box for transport to Denmark. Before deploying the freshwater pearl mussels in the stream channels, they were acclimated to stream channel conditions in darkness at 10°C. The acclimation was conducted using plastic buckets containing aerated tap water. After 24 h of acclimation, 19 mussels were released into each of five of the stream channels within a 3-m section located closest to the inlet.

Empty shells from freshwater pearl mussels were collected from Brons stream in central Sweden (62°37′ N, 17°43′ E). The population of freshwater pearl mussels in Brons stream was severely devastated in the 1990s following a disease eradicating most of the population (Håkan Söderberg, Länsstyrelsen, Sweden, personal communication). Hence, empty shells were assumed to originate from individuals dying during this catastrophic event 20–30 years ago. The shell material was collected by hand the day before collecting living individuals in Hemgravs stream and transported to Denmark in cooling boxes. Empty shells were evenly distributed with 19 individual shells in each of the five channels.

2.3 | Experimental phase and measured environmental variables

Substrates were added to the channels in the first week of October 2019, and subsequently the central feeding pump was activated providing stream water for the channels. Discharge was maintained for 5 weeks enabling conditioning of microbial communities on the substrates.

Living freshwater pearl mussels and empty shells were added to the stream channels on 11 November 2019. Apart from the five channels containing live mussels and five containing shells, two channels remained empty serving as negative controls. One water sample (1.5 L) was collected from each channel for eDNA analysis, and the sampling was repeated at weekly intervals for the subsequent 3 weeks. In total, 36 water samples were collected throughout the experiment. The water sampling was conducted in the downstream end of the channel, first in negative control channels, then in channels with shell material, and finally in channels containing living individuals.

Current velocity was measured at each water sampling event in the upstream end of each channel using a Höntzsh current velocity meter. Probes and wires were disinfected using 0.5% bleach and 70% ethanol between measurements in each channel. Temperature and pH were measured in water collected from the channels in 5-L buckets at each water sampling event using a pH meter (YSI, Yellow Springs, OH, USA). New buckets were used for each channel to avoid cross contamination. Water chemistry parameters were not measured in this study, but an overview of approximate levels of nitrate-N, ammonium-N, total N, ortho-phosphate-P, and total P were obtained from previous studies in the stream channel facility (Graeber et al., 2017; Neif et al., 2017). Lemming stream, which supplies water to the stream channels, has a small catchment dominated by forest, and nutrient levels have been consistently low during the past 2 decades (total N and total P concentrations ~1 mg L^{-1} and 0.01 mg L^{-1}, respectively, data extracted from the Danish open source database for aquatic monitoring data: https://oda.dk). Successful mimicking of environmental conditions at the sampling

sites for the test organisms should ensure minimum organismal stress in the mesocosm channels. This is important as stressed organisms may shed DNA at different rates compared with the natural scenario.

At the end of the experiment, all living individuals and all empty shells were collected. Height, width, shell thickness, and wet weight (WW) of all living individuals were measured using a vernier caliper. Only WW of empty shells was measured, as these were too porous to measure their size without breaking. The WW of empty shells was measured for all shells in three of the stream channels. At the end of the experiment, all living individuals and empty shells were frozen.

2.4 | Water sample processing

The sampling was conducted in the downstream end of each stream channel using sterile disposable plastic cups and bags (2 L) (Oriplast Krayer GmbH, Neunkirchen, Germany). Collected water was immediately filtered (Millipore Sterivex filter, 0.22 μm, Merck KGaA, Darmstadt, Germany) using the Pressure Assisted Filtration System developed by NIRAS A/S and modified from Hesselsoe et al. (2015). To avoid broken filter membranes, this method ensured a constant maximum pressure on the filter membrane equivalent to the specifications from the supplier (3 bar). As the sample water did not come into contact with the filtering apparatus, cleaning of the apparatus between samples was not necessary. Disposable nitrile gloves were worn during sampling and replaced between sampling of each channel. Filtering was performed for a maximum of 15 min or until the filter clogged. The remaining water was removed from the filters using a 60-ml disposable sterile syringe (BD Plastipak, USA) and then closed using sterile stoppers in both ends of the filter. All filters were immediately stored on dry ice until DNA extraction and analysis. To minimize cross contamination, sampling was conducted in the same order at each sampling event starting with the negative control channels, followed by the channels with empty shell material, and finally in the channels containing living individuals.

2.5 | Species-specific quantitative polymerase chain reaction system

The eDNA probe assay for species-specific detection of *M. margaritefera* consists of a forward primer MmarCOI-F: 5′-TTG CTG AGC GAA TTC CTT TGT TC-3′, reverse primer MmarCOI-R: 5′-CCC-TGC CAA AAC CGG TAG TG-3′, and a 5′-6-FAM labelled TaqMan probe MmarCOI-P: 5′-CCA CTA ACA AAA TAG CCG TCA CCG TAA CA-3′, targeting the cytochrome oxidase I (COI) region of the mitochondrial genome. The length of the produced amplified product is 83 bp. The assay was developed using Geneious v10.2.3 (http://geneious.com, Kearse et al., 2012) and customized to avoid cross amplification of the most relevant non-target species. The species *Unio pictorum* (JX046561.1), *Unio crassus* (JX046553.1), *Unio tumidus* (GU230750.1), *Anodonta anatina* (KY328508.1), *Anadonta cygnea* (JQ253887), *Pseudanodonta complanata* (JQ253892.1), and *Margaritifera auricularia* (AF303309.1) were selected as non-target oligos as they are commonly found in Scandinavia and are closely related to *M. margaritifera*. The oligos were designed to cover as many single nucleotide polymorphisms (SNPs) as possible from the non-target species placing some of the most common SNP at the 3′ end of both forward and reverse primers to maximize destabilization and hence specificity of the assay (see Figure S1). A BLAST search was performed using the target polymerase chain reaction (PCR) amplicon sequence. It returned 51 target sequences from *M. margaritifera*, of which 49 had a total match with the oligos. The remaining two sequences, KY775536 and KU763232, had one SNP in the reverse primer which probably did not affect the assay substantially as it is located in the 5′ end of the primer.

Further, *in-silico* specificity assessment of the oligos showed sequence full crossmatch (0 SNPs) for the forward primer with *Pletholophus renianus* (MT020604), *Sinanodonta calipygos* (MT020624), and *Cristaria tenuis* (MT020599). However, the reverse primer and the probe showed the presence of a total 11 or 12 SNPs with these organisms, preventing any chances of proper amplification (Table S1).

In total, 36 non-target bivalve species were identified with the BLAST search (see overview in Table S1). Alignments showed a total number of SNPs against the oligos varying from six (*Ensidens sagittarius*) to 17 (*Pleurobema cordatum*). Inspection of the sequences with the lowest number of SNPs shows the critically important presence of SNPs at the 3′ end position of the primers, indicating strong specificity of the assay for *M. margaritifera*.

In vitro amplification in non-target species was tested against tissue samples from brown trout, thick shelled river mussel (*Unio crassus*), duck mussel (*Anodonta anatina*), and swan mussel (*Anadonta cygnea*). These three dominant species of large mussels in Norway, in addition to *M. margaritifera* (Lopes-Lima et al., 2017), showed an accumulated number of SNPs of 14 for *A. anatina* and 16 for *A. cygnea* and *U. crassus* (Table S1).

For optimization and testing of the detection system a Bio-Rad CFX96 Touch (Bio-Rad, Hercules, CA, USA) was used for quantitative PCR (qPCR) in a 25-μl reaction volume, consisting of 500 nM of each primer and 200 nM probe, 2.5 μl DNA sample, and 12.5 μl TaqMan Environmental Master Mix 2.0 (ThermoFisher Scientific, Waltham, MA, USA). The thermocycling two-step protocol consisted of an initial warming at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. All samples were run in duplicates from the same PCR Mastermix during optimization and included both positive and negative controls.

2.6 | eDNA analyses of water samples

Environmental DNA was extracted from the filters using the Qiagen DNeasy® Blood and Tissue Kit (modified spin column protocol) as described by Knudsen et al. (2019). Final elution volume was 50 μl. Protocol and thermal settings for qPCR followed the descriptions above (Section 2.5) except that 5 μl of DNA sample was added to

each PCR reaction with a final volume of 25 μl. All samples were analysed in triplicate. An intern positive standard was added to all PCRs to monitor PCR inhibition (TaqMan™ Exogenous Internal Positive Control Reagents, ThermoFisher Scientific).

2.7 | eDNA controls and standard curves

The qPCR analyses were performed in strips of eight PCR tubes including two no-template controls (NTC). These were included to estimate the probability of false positives. It is common practice to monitor false positives using negative controls (NTC) whenever using PCR amplification. If amplification was observed in one or more NTC, the results of the analyses were excluded and repeated. Often, very few negative controls are included in the PCR-setup; two to four per plate with 96 samples (i.e. <5%). However, the probability that contamination will be detected in exactly those two to four NTCs is low. This implies that a potential contamination leading to a false positive may remain concealed. Hence, few NTCs (i.e. <5%) are not always sufficient to discover false positives due to contamination. Consequently, the probability of false positives was estimated assuming an equal risk of contamination for NTCs and the analysed samples. Based on the number of negative NTCs on each PCR plate, the probability of false positives among the samples analysed can be calculated (Sørensen et al., unpublished data).

2.8 | Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) were assessed from standard curves using dilution series based on DNA extracted from *M. margaritifera* tissue. Three replicates were carried out for each dilution step following the MiQE principles (Bustin et al., 2009; Mauvisseau et al., 2019). LOD was defined as the last standard dilution where at least one of the three technical replicates gave a positive detection while LOQ was defined as the last standard dilution where all three technical replicates were positive and within the same range. LOD and LOQ were estimated for qPCR setups (96 PCR wells) and consequently differed slightly between the set-ups.

2.9 | Data treatment

Individual sizes (length, height, shell thickness, and WW) of living individuals were compared among channels ($n = 5$) using a one-way analysis of variance (ANOVA). Homogeneity of variance and normally distributed data were tested using Kolmogorov–Smirnov tests and Levene's test, respectively ($\alpha = 0.05$).

As pH, temperature, and current velocity may influence the eDNA signal (Barnes et al., 2014), tests were carried out for significant differences among sampling dates ($n = 12$) and among treatments (living individuals vs. shell material; $n = 5$) for these parameters. Potential differences in pH, temperature, and current velocity between treatments were tested using a paired t-test, while differences among sampling dates were tested using one-way ANOVA. Test assumptions for homogeneity of variance and normally distributed data were checked using Kolmogorov–Smirnov and Levene's tests, respectively ($\alpha = 0.05$).

3 | RESULTS

3.1 | Environmental parameters

No significant differences were observed in current velocity, pH, or discharge among treatments (living individuals vs empty shells, paired t-test, $P > 0.05$, $n = 5$) or in current velocity among sampling dates (one-way ANOVA, $P > 0.05$, $n = 12$; Table 1). However, pH and temperature varied significantly among sampling dates (one-way ANOVA, $P < 0.0001$, $n = 12$) with lowest temperatures and highest pH occurring at the first and last sampling dates (Table 1).

3.2 | Sizes of living freshwater pearl mussels and empty shells

Individual sizes of living individuals were not significantly different among channels in length, width, height, or WW (one-way ANOVA, $P > 0.05$, $n = 19$, Figure 1). Similarly, no significant differences among average individual WW of the shell material for one channel were found (one-way ANOVA, $P > 0.05$, $n = 19$; data not shown).

3.3 | Environmental DNA analyses

LOD from the qPCR set-ups ranged between $Ct = 39.4$ and 39.7, while LOQ ranged between $Ct = 35.6$ and 36.6. No traces of eDNA from freshwater pearl mussels were found in channels with empty shells, whereas eDNA was detected in all but one sample originating from channels with living individuals (Table 2). All three technical replicates were negative for that one sample. The eDNA concentrations in water samples from channels with living individuals tended to decrease (increasing Ct values) with time (Table 2). No traces of eDNA from freshwater pearl mussels were found in samples from the negative control channels except for the sample collected in channel No. 11 in experimental week 1 (Table 2). One of the three technical replicates was positive for eDNA for that one sample.

3.4 | Uncertainty estimates

In total, 108 qPCR analyses were conducted on samples collected from the stream channels, 42 qPCR analyses on samples for producing a standard curve, and 126 NTCs. Three technical replicates were analysed per sample. The results showed that 75 of those were positive and 75 were negative. Together with the 126 negative

TABLE 1 Environmental parameters measured during the experiment presented as average values (±SE)

	Experimental week					
	1		2		3	
Treatment group	Empty shells	Living individuals	Empty shells	Living individuals	Empty shells	Living individuals
Discharge (L s^{-1})	1.92 (±0.21)	1.36 (±0.21)	1.70 (±0.18)	1.81 (±0.27)	1.59 (±0.22)	1.86 (±0.27)
Temperature (°C)	6.44 (±0.05)	6.42 (±0.04)	7.70 (±0.02)	7.66 (±0.02)	6.04 (±0.02)	6.04 (±0.02)
pH	7.51 (±0.01)	7.50 (±0.00)	6.93 (±0.02)	6.92 (±0.02)	7.40 (±0.02)	7.38 (±0.02)

Note: Each average value represents one sampling event for one treatment (i.e. living freshwater pearl mussels and empty shells; n = 5). Three sampling events were included in the 3-week experimental period (from mid-November to early December 2019).

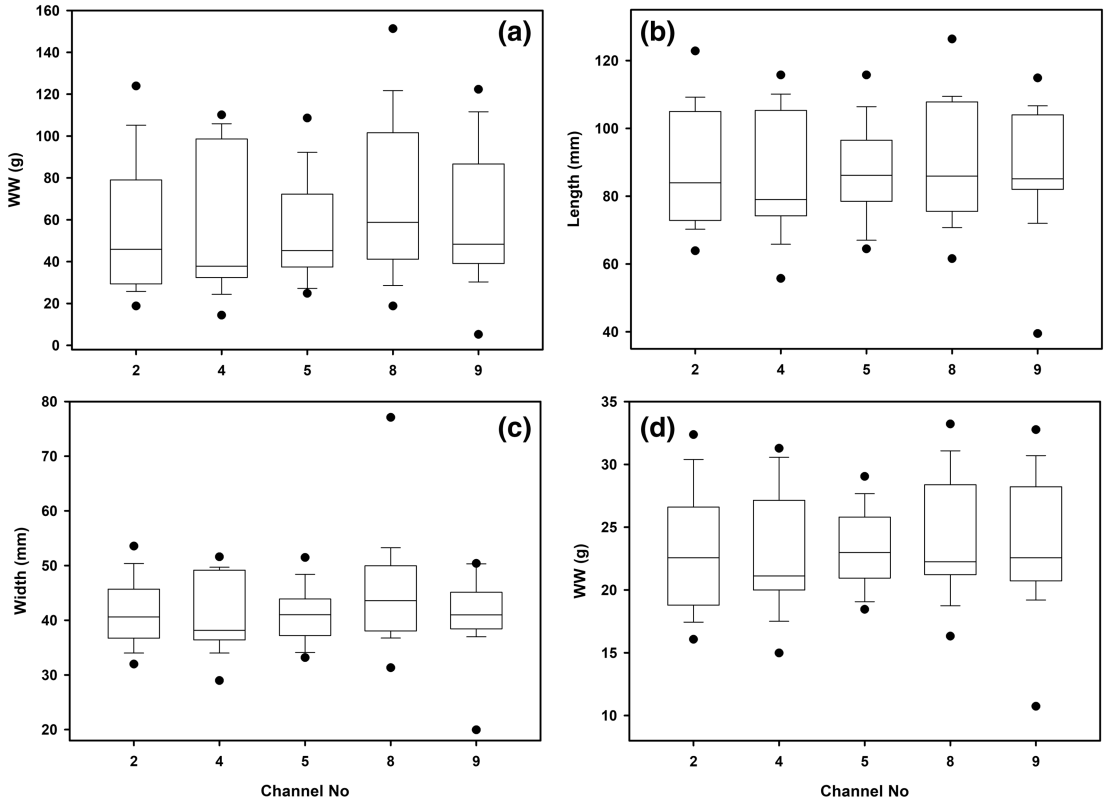

FIGURE 1 Boxplots showing wet weight (WW; a), length (b), width (c), and height (d) of living freshwater pearl mussels used in the experiment for each replicate channel. Upper and lower edges of each box represent 25 and 75 percentiles, respectively. Error bars represent 10 and 90 percentiles, respectively. The median value is indicated as a solid line inside each box. Outliers are shown

controls (all were negative as expected) the probability of producing a false positive was estimated at 1.1% given these conditions.

4 | DISCUSSION

4.1 | Environmental DNA from empty shells and living freshwater pearl mussels

All water samples from stream channels containing empty shells from *M. margaritifera* were negative for eDNA. This indicates that eDNA traces detected in stream water with high probability originate from living individuals located upstream of the sampling site. Hence, the findings by Geist, Wunderlich & Kuehn (2008), who showed that DNA could be extracted from empty shell material from *M. margaritifera*, should not be considered as proof that there is a significant risk of false positives in the field. Geist, Wunderlich & Kuehn (2008) ground up empty shells from recently dead freshwater pearl mussels (<3 months since death) with the sole purpose of extracting DNA from mussels in a non-destructive way. Furthermore, chloroform or ethanol was applied to facilitate DNA extraction from the shell material (Geist, Wunderlich & Kuehn, 2008). The shell material collected remained intact throughout the experimental period and had an estimated age of 20–30 years. Consequently, we conclude that shell material originating from *M. margaritifera*, dead in the range of decades, does not secrete DNA in quantities that can be measured

TABLE 2 Results overview from the quantitative polymerase chain reaction analyses based on water samples collected from the stream channels

Channel No.	Treatment	Experimental week		
		Week 1	Week 2	Week 3
7	Negative control	No Ct[b]	No Ct[b]	No Ct[b]
11	Negative control	37.96[a]	No Ct[b]	No Ct[b]
2	Living individuals	33.95	36.58	38.40
4	Living individuals	No Ct[b]	38.10	37.75
5	Living individuals	35.31	35.94	37.42
8	Living individuals	36.04	37.64	37.48
9	Living individuals	34.24	35.87	38.06
1	Shell material	No Ct[b]	No Ct[b]	No Ct[b]
3	Shell material	No Ct[b]	No Ct[b]	No Ct[b]
6	Shell material	No Ct[b]	No Ct[b]	No Ct[b]
10	Shell material	No Ct[b]	No Ct[b]	No Ct[b]
12	Shell material	No Ct[b]	No Ct[b]	No Ct[b]

Note: Ct values are presented for each channel and sampling date (experimental weeks 1–3). Each Ct value represents an average based on three technical replicates.

[a]Only one of the three technical replicates positive (not reproducible).

[b]Ct denotes the number of amplification cycles in qPCR required to detect the target DNA. No Ct indicates no detectable DNA.

with current analytical techniques. Note, however, that recently dead individuals, especially if degrading soft tissues still exist, probably excrete DNA, potentially leading to false positive results.

Water samples collected from stream channels containing living individuals of *M. margaritifera* (density ~10 individuals m^{-2}) were consistently positive for eDNA except for one sample (all technical replicates negative). The eDNA concentration was even above the LOQ for several samples, clearly indicating that the applied experimental set-up and population density of *M. margaritifera* as well as the analytical methods were suitable for the purpose of the study. As we did not find any significant differences for abiotic (pH, current velocity, and temperature) and biotic (size and weight of individuals) parameters among channels containing living individuals, we assume that the one negative sample was a false negative caused by sampling, storage, DNA extraction, or other analytical bias prior to the PCR analysis. One false positive replicate was also recorded in one of the channels without shells. As this observation in one out of three replicates was not reproduceable, the sample analysed was not considered positive. Despite a low estimated probability of a false positive, this emphasizes the importance of tracking contamination risks using a high number of NTCs.

The eDNA concentrations in stream channels containing living individuals decreased with time (increasing Ct values). This finding was interpreted as an initial increased DNA secretion resulting from stress imposed on the mussels during collection, transport, acclimation, and deployment in the channels followed by slowly normalizing DNA secretion towards lower levels as the mussels established their natural position and behaviour in the channel sediments (Stewart, 2019). Equivalent findings have been achieved in mesocosm experiments using fish, showing an initial spike in eDNA concentration before declining over time (Barnes et al., 2014) due to initial stress (Maruyama et al., 2014; Sassoubre et al., 2016). In addition, secretion and persistence of eDNA may also be significantly influenced by temperature (Barnes et al., 2014; Strickler, Fremier & Goldberg, 2015) and pH (Strickler, Fremier & Goldberg, 2015; Seymour et al., 2018), and significant changes in temperature and pH were observed during the present study. However, the changes were not temporally systematic, as the first and last sampling events were characterized by lower temperature and higher pH compared with the intermediate sampling event. Consequently, it is not likely that the variation in temperature and pH was an important factor governing the temporally consistent decreasing eDNA concentrations.

4.2 | Perspectives

Being one of the most endangered freshwater mussels, *M. margaritifera* is considered a high conservation priority among countries hosting the remaining populations (Geist, 2010). Owing to pragmatic constraints (e.g. costs and time allocation) affiliated with conventional surveys aiming to describe distributions of remaining populations, eDNA has been used increasingly as a promising alternative or supplementary method for performing such surveys in a more cost-efficient way (Sousa et al., 2015; Stoeckle, Kuehn & Geist, 2016; Wacker et al., 2019). Especially in deep and turbulent streams with low visibility, conventional surveys may benefit profoundly from this method. Note, however, that eDNA methods mainly serve the purpose of identifying presence or absence of individuals (i.e. population distributions) as they fall short in terms of quantifying population size, age distribution, and reproductive potential, which are all vital elements for assessments of conservation status.

This study shows that positive eDNA signals for *M. margaritifera* probably reflect the presence of living individuals (or recently dead ones with actively degrading soft tissues). Local distributions of *M. margaritifera* (on the 1–10 m reach scale) often show highly accumulated/condensed patterns, probably reflecting subtle variations in substrate and flow characteristics affiliated with substrates comprising stones, boulders, and sand (Boycott, 1936; Hastie, Boon & Young, 2000). As Stoeckle, Kuehn & Geist (2016) showed that downstream transport of eDNA from freshwater pearl mussels was restricted to <500 m in lowland streams, the eDNA technique provides a useful tool to restrict the search for local, small, and scattered populations to a few hundred metres. Note, however, that transport distances can exceed 1,700 m in stream systems with low retention (i.e. a long spiralling length) such as mountain streams dominated by stones and cobbles (Wacker et al., 2019). Conversely, when population sizes are small (i.e. comprising only a few individuals), water samples may need to be collected within a

few metres of the individuals owing to the dilution factor of running water systems (Gasparini et al., 2020). Therefore, water sampling strategies may have to be tailor made for specific stream typologies and the sizes of resident populations.

It is well established that eutrophication and especially habitat degradation are strong drivers of the severe and continuing population decline of freshwater mussels in general (Bogan, 2008; Haag & Williams, 2014) and *M. margaritifera* in particular (Geist, 2010). Although adult individuals of *M. margaritifera* can probably survive sub-optimal habitat conditions for longer periods, the juveniles are much less tolerant towards habitat changes and impairments. As such, the juveniles require consistently high substrate stability, and show limited tolerance to excessive sedimentation of fine particles (Boycott, 1936; Hastie, Boon & Young, 2000). Even simple physical and chemical stream habitat surveys in deep and turbulent streams can quantify temporal variability and habitat suitability for *M. margaritifera* within stream sections of a few hundred metres. Even in the absence of detected living individuals of *M. margaritifera* in such deep and turbulent lowland streams, positive eDNA samples can both be interpreted as a reasonably reliable prediction of the presence of living individuals, as well as a strong indication that these individuals are located in close upstream proximity of the sampling point. Consequently, we urge that eDNA sampling should be used to locate remaining population fragments in deep and turbulent streams, and that physical and chemical habitat surveys should be executed at sampling sites with positive eDNA samples providing a platform for faster and more efficient decision making when launching mitigation initiatives. For streams with several unionid species, metabarcoding approaches could be applied as alternatives to species-specific detection systems (Klymus et al., 2021). In addition, the presence of freshwater pearl mussel DNA in lowland stream water could be used to guide more specific search initiatives targeting visual identification of the remaining small and scattered populations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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