

# Using environmental DNA to monitor the reintroduction success of the Rhine sculpin (*Cottus rhenanus*) in a restored stream

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## Abstract

As a consequence of the strong human impact on freshwater ecosystems, restoration measures are increasingly applied to restore and maintain their good ecological status. The ecological status of freshwaters can be inferred by assessing the presence of indicator species, such as the Rhine sculpin (*Cottus rhenanus*). However, traditional methods of monitoring fish, such as electrofishing, are often challenging and invasive. To augment or even replace the traditional fish monitoring approach, the analysis of environmental DNA (eDNA) has recently been proposed as an alternative, sensitive approach. The present study employed this modern approach to monitor the Rhine sculpin, a species that has been reintroduced into a recently restored stream within the Emscher catchment in Germany, in order to validate the success of the restorations.

We monitored the dispersal of the Rhine sculpin using replicated 12S end-point PCR eDNA surveillance at a fine spatial and temporal scale to investigate the applicability of analyzing eDNA for freshwater ecosystem monitoring. We also performed traditional electrofishing in one instance to compare visual and eDNA-based assessments.

We could track the dispersal of the Rhine sculpin and showed a higher dispersal potential of the species than we assumed. Furthermore, the eDNA analysis showed higher sensitivity for detecting the species than traditional electrofishing, although false negative results occurred at early reintroduction stages. Our results show that analyzing eDNA is capable of validating and tracking ecological reintroductions and contribute to the assessment and modelling of ecological status of streams.

## Introduction

Freshwater ecosystems are heavily impacted due to habitat fragmentation caused by wetland drainage, river straightening and dam building in combination with poor water quality caused by agricultural and industrial pollution (Jensen, Trepel, Merritt, & Rosenthal, 2006). The determination of their ecological status is hence an important task to assess if restorations are needed for restoring and maintaining a good status of these ecosystems. The ecological status of a freshwater ecosystem can be inferred by assessing indicator species, which include algae, benthic macroinvertebrates and fish (Bellinger & Sigeo, 2015; Karr, 1981; Resh & Unzicker, 1975).

The Rhine sculpin (*Cottus rhenanus*; Freyhof, Kottelat, & Nolte, 2005) and the European bullhead (*Cottus gobio*; Linnaeus, 1758) are two closely related freshwater sculpin species which prefer similar ecological habitats. They are used as such fish indicator species for water quality, structure and passage of flowing waters as they require well-oxygenated streams (Colleye, Ovidio, Salmon, & Parmentier, 2013) with gravelly to stony stream beds (Wittkugel, 2005), show a stationary behavior with limited home ranges (Ovidio, Dettaille, Bontinck, & Philippart, 2009) and are presumed to be incapable of crossing barriers higher than 18 cm (Uttinger, Roth, & Peter, 1998). The Rhine sculpin used to be resident in the Emscher catchment, North Rhine-Westphalia, Germany, but became locally extinct in the 19<sup>th</sup> century when the catchment was used as an open sewer system for wastewater disposal (Brink-Kloke et al., 2006). The species only survived in one tributary stream, which was less anthropogenically impacted during that period (Donoso-Büchner, 2009). Today, one of the largest European infrastructure projects supports comprehensive river restructuring of the Emscher catchment, with 4.5 billion Euro invested in the project as of 2015 (Böhmer, 2015). The project aims to restore the river and all its tributaries to a near natural ecological status (Schnelle & Wilts, 2016). These efforts make it possible to reintroduce individuals from the isolated Rhine sculpin population back into the restored streams to validate the restoration success.

Monitoring the success of reintroducing the Rhine sculpin is however logistically challenging. Electrofishing, where fish are temporarily stunned using an electronic device, is the established method for collecting individuals. This traditional approach is invasive for ecosystems and not always feasible (Bohlin et al., 1989; Platts, Megahan, & Minshall, 1983). The application of environmental DNA (eDNA) is a promising, non-invasive alternative for the detection and

monitoring of species in aquatic environments (*reviewed in* Thomsen & Willerslev, 2015). While eDNA analysis comes with several limitations (*reviewed in* Goldberg, Strickler, & Pilliod, 2015), recent studies have shown its potential to infer fish diversity in streams, lakes and the ocean (Dejean et al., 2012; Hänfling et al., 2016; Jerde, Mahon, Chadderton, & Lodge, 2011; Miya et al., 2015; Takahara, Minamoto, & Doi, 2013; Yamamoto et al., 2016). A recent large-scale comparison has shown reliability and higher success rates when inferring the presence of fish species in rivers via eDNA as compared to electrofishing (Pont et al., 2018).

The potential of eDNA for monitoring fish dispersal has been shown for invasive species (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Laramie, Pilliod, & Goldberg, 2015; Takahara et al., 2013), however, it has not yet been applied to investigate the dispersal of ecologically reintroduced fish species. Therefore, the aim of this study was to use eDNA for monitoring the reintroduction success and dispersal of the Rhine sculpin in a restored stream of the Emscher catchment. In the context of this study, the recently restored stream Borbecker Mühlenbach within the Emscher catchment was deemed suitable for the Rhine sculpin again post restoration. The species was therefore reintroduced to further validate the stream's good status after its restoration. We monitored the fish's dispersal in a high spatial and temporal resolution to test three specific hypotheses: (1) The reintroduced Rhine sculpin individuals will disperse faster upstream than expected from the species' typical stationary behavior (maximum of 149 m in 27 days in an established population = 5.51 m/day; Ovidio et al., 2009) due to the high density of individuals at the reintroduction sites; (2) the reintroduced individuals are not able to cross a potential dispersal barrier and will not be detected upstream of that barrier; and (3) individuals will establish along the whole stream section until the dispersal barrier due to the habitat's expected suitability for the species. As a comparison between eDNA-based and traditional monitoring, we additionally carried out electrofishing to validate our results.

## Material and Methods

### *Field site*

The Borbecker Mühlenbach is a small urban stream categorized as German stream type 6 (fine-grained, carbonic mountain streams, Sommerhäuser & Pottgiesser, 2005; Fig. 1 A), with its source in Essen, North Rhine-Westphalia, Germany. Like most streams within the Emscher catchment, the Borbecker Mühlenbach was used as an open sewer system for wastewater disposal from the 19<sup>th</sup> century on but was recently restored (mainly carried out in 2011, finished in 2014) and today consists of both piped underground sections and restored above ground sections. This study focuses on an above ground section of the Borbecker Mühlenbach, which represents a young freshwater ecosystem due to its recent restoration. Within the studied stream section, a loose stone dam serves as a water barrier and potential dispersal barrier for the Rhine sculpin (Fig. 1 B). Further upstream the stream Kesselbach enters the Borbecker Mühlenbach. After the stream had been deemed suitable for the Rhine sculpin again post restoration, 118 Rhine sculpin individuals taken from the river Boye (Bottrop, Germany) were reintroduced into the stream at sampling sites 3-5 (Fig. 1 C) on the 23<sup>rd</sup> of August 2017. To prevent individuals from drifting downstream into underground pipes, a net was installed in front of the pipes.

### *Sampling*

Water samples for subsequent eDNA analyses were taken along the 1050 m long restored above ground section of the stream. 14 sites were sampled on the first two sampling days, 2 and 5 days after introduction of the fish, respectively, and 15 sites on the following sampling days (Fig. 1; sampling site 1a was added 50 m behind site 1 after two sampling days). Sampling was carried out every fifth day for 15 days, then once a month for three months. A final sampling day was carried out after one year, which included both eDNA collection and electrofishing. Due to unexpected findings, additional samples were taken after one additional month from site 10 on (sampling sites 11a and 11b were added respectively 50 m behind site 11). Prior to the reintroduction of the Rhine sculpin, the stream was sampled every 200 m to take negative control samples. Sampling dates and names are shown in Fig. 2.

For every eDNA sample, 1 L of water was collected in bottles (sterilized in 4% chlorine bleach overnight after each use) and filtered through sterile 0.45  $\mu\text{m}$  cellulose nitrate filters (Thermo Scientific™ Nalgene™ filtration units, Thermo Fisher Scientific, Waltham, USA) using a vacuum pump. The filtering took place in the field to prevent cross-contamination by other laboratory samples. For every 500 ml, a separate filter was used (2 filters per sample). Additionally, each sampling day an extra filter was exposed to air and then included with the other samples. This was done to check for cross-contamination by air on site (blank sample). The filters were then preserved in 96% ethanol and stored at  $-20^{\circ}\text{C}$  until extraction.

Additionally, on the 5<sup>th</sup> of November 2018, electrofishing was carried out after taking water samples for eDNA analyses. Every sampling site was electrofished for a stretch of 20 m in upstream direction from the point of eDNA sampling, apart from sites 11a and 11b, which were added later on, and from site 14, which was not accessible for electrofishing due to the presence of dense vegetation.

### *Laboratory work*

All lab work was carried out in an eDNA laboratory with 45 mins of UV-light sterilization between separate work cycles. DNA was extracted using a modified salt extraction protocol (original protocol by Sunnucks & Hales, 1996; for modified protocol see Weiss & Leese (2016), Additional file 2). For the purification, either the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) or the MinElute Reaction Cleanup Kit (QIAGEN Inc., Germantown, Maryland, USA) were used, following the manufacturer's instructions. The final DNA extract was eluted into 20  $\mu\text{L}$  of water. The success of all extractions and purifications was verified using agarose gel electrophoresis.

Primers targeting the hypervariable mitochondrial 12S rRNA gene of the Rhine sculpin were designed using a reference database containing 272 sequences of the 12S rRNA gene from 57 fish species (Hänfling et al., 2016) including all native fish species resident in the Borbecker Mühlenbach and Kesselbach. The complete mitochondrial genome of the Rhine sculpin was downloaded from NCBI GenBank (MF326941/NC\_036147, both identical), which was representative of the study population in the 12S rRNA gene sequence, and added to the

downloaded 12S rRNA dataset (for final mafft alignment (Katoh & Standley, 2013) of 12S rRNA gene sequences see Supplementary Data S1).

The presence of Rhine sculpin DNA within the eDNA samples was tested with nested end-point PCRs using the QIAGEN Multiplex PCR *Plus* Kit (QIAGEN Inc., Hilden, Germany). For the first PCR step, 2  $\mu$ L of the extracted DNA, 0.25  $\mu$ L of 100  $\mu$ M universal fish 12S rRNA primers 12S\_30F and 12S\_1380R (Hänfling et al. 2016), 25  $\mu$ L of the Multiplex MasterMix and 22.5  $\mu$ L water were used per reaction. PCR conditions consisted of an initial incubation at 95°C for 15 minutes followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 50 seconds, and a final elongation at 72°C for 10 minutes. For the second PCR step, 1  $\mu$ L template of each PCR product from step 1, 0.25  $\mu$ L of 100  $\mu$ M newly designed primers C\_12S\_377F (5'–AGGCCCAAGTTGACAAACAC–3') and C\_12S\_731R (5'–GGCGGGTAAACAAGGAAGG–3'), 12.5  $\mu$ L of the Multiplex MasterMix and 11.25  $\mu$ L water were used per reaction. PCR conditions consisted of an initial incubation at 95°C for 15 minutes followed by 30 cycles of 95°C for 20 seconds, 63°C for 30 seconds, and 72°C for 1 minute, and a final incubation at 72°C for 5 minutes. The newly designed primers amplify a 344 bp long region of the Rhine sculpin's 12S rRNA, which is located within the 12S section targeted by the universal fish 12S rRNA primers used in step 1. The designed primer pair is universal for 20 out of 24 species of the *Cottus* complex with available 12S sequences on GenBank including Baikal sculpins according to PrimerBlast (NCBI, <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Due to the presence of only the Rhine sculpin in the investigated stream, misamplification of other *Cottus* species caused by the universality of the primer pair was excluded.

Half of the extracted DNA of every sample (10  $\mu$ L) was used as template for the first PCR step to minimise stochasticity effects, leading to five PCR replicates per sample with 2  $\mu$ L of the extracted DNA each. The presence of the Rhine sculpin was confirmed as detected in a sample if at least one of the five reactions of the second step showed a visible band at expected amplicon length using agarose gel electrophoresis with 1% agarose gels run for 15 minutes at 80 V. In case the Rhine sculpin was not detected in the first half of the eluate, the remaining eluate was used as an input for further PCRs. If this approach also did not show any signal, then the Rhine sculpin was considered to be absent in a sample.

183 Samples from each sampling day were tested up to site 12. If the species was detected behind the  
184 barrier (sites 11 and 12), then sites 13 and 14 were also tested.

185 To confirm the specificity of the primer pair, PCR amplicons of eleven positive replicates from  
186 different samples were Sanger sequenced (Eurofins Genomics, Ebersberg, Germany), which all  
187 confirmed the identity of the Rhine sculpin.



## Results

No DNA was found in the blank controls, excluding cross-contamination during filtering on site and confirming clean DNA extraction and purification. Moreover, no DNA was found in any negative control of the nested end-point PCRs, confirming clean PCR setup.

The Rhine sculpin was not detected in any negative control sample, confirming its absence in the stream section prior to the reintroduction (Fig. 2). Within the first three months, the Rhine sculpin was detected at least once at every sampling site before the barrier (site 1-10) and not detected behind the barrier (site 11 and 12). Sample 9 *B* and 10 *C* were positive, confirming the fish's dispersal of 200 m upstream within the first five days and 250 m within the first ten days, respectively. However, the species was not detected in several samples despite the presence of the species further upstream on the same day. In general, positive detections strongly varied among technical replicates, from five detections in five replicates to one detection in ten replicates. Positive detections in all technical replicates of one sample were rare, indicating low DNA template concentration in the stream.

After one year, the species was detected at every sampling site before the barrier, with positive detections in all technical replicates of each sample (apart from sample 4 *G*, of which only four of five technical replicates were positive). In addition, the Rhine sculpin was detected at sites 11 *G* and 13 *G* behind the barrier. In contrast, with electrofishing the fish was detected at every site before the barrier (apart from site 1) but not behind the barrier. A total number of 113 individuals were found, including juveniles (Fig. 2).

After one additional month, the species' presence behind the barrier was confirmed by eDNA detection at sites 11 *H*, 11a *H* and 11b *H* and Sanger sequencing (Fig. 2).

## Discussion

### *Dispersal of the Rhine sculpin*

The increasing number of eDNA-based detections of the Rhine sculpin over time combined with the visual observation of individuals via electrofishing, including juveniles, indicated reproduction. This further showed successful reintroduction, dispersal and establishment of the species within the stream Borbecker Mühlenbach. Furthermore, the species' presence indicates good water quality, oxygenation and habitat structure of the stream and hence a successful stream restoration.

Based on end-point PCR eDNA detection, we showed that individuals dispersed 200 m within the first five days (positive sample 9 B), on average 40 m/day, which greatly exceeds daily moving distances observed for the Rhine sculpin in an established population by Ovidio et al. (2009; maximum of 5.51 m/day). This verifies our first hypothesis that the reintroduced individuals disperse faster from their reintroduction sites than expected from their known stationary behavior. A possible explanation for this pattern is that the initially high density of individuals at each reintroduction site led to high intraspecific competition. Therefore, a density-dependent behavioural mechanism to compensate for this is the rapid dispersal away from the point of reintroduction. However, it is notable that maximum moving distances of up to 395 m for the Rhine sculpin within one month and over 250 m for the closely related European bullhead within several months have been observed (Knaepkens, Baekelandt, & Eens, 2005; Knaepkens, Bruyndoncx, & Eens, 2004; Ovidio et al., 2009). Furthermore, seasonal migration has been shown for the European bullhead (Crisp, Mann, & Cubby, 1984). However, none of these studies investigated the fish's movement at a temporal scale comparable to the present study.

The Rhine sculpin was not detected in eDNA samples upstream from site 10 within the first three months and furthermore not detected with electrofishing after one year, indicating a dispersal limitation by the barrier before site 11. However, eDNA detections of the species behind the barrier after one year (detections at sites 11 G and 13 G) and moreover after one additional month (detections at sites 11 H, 11a H and 11b H) show that at least some Rhine sculpin individuals were able to cross the barrier. This result was unexpected (see second hypothesis) and not supported by electrofishing investigations. However, we based this hypothesis on the finding that sculpins are unable to cross stream barriers of 18 cm height (Utzinger et al., 1998), whereas the barrier in the

present study consists of loose stones and is occasionally flooded during heavy rainfall events. The species' dispersal across the barrier therefore might not confirm its ability to cross high barriers, but nevertheless indicates a higher dispersal potential of the species than previously assumed, as the loose stone dam represents a substantial dispersal barrier for small, benthic fish. The possibility that juveniles were able to swim through the dam should be further investigated. These findings confirmed that there was good passage throughout the stream. However, as the stream is urban and frequently visited by pedestrians, human-induced dispersal across the barrier cannot be excluded. The inconsistency between the eDNA-based and traditional monitoring indicates that individuals were located upstream of the electrofishing site and hence could not be detected via electrofishing. However, they were still releasing eDNA which was transported downstream and collected, making the detection of the individuals possible.

The third hypothesis that individuals establish along the whole stream section up to the barrier due to the habitat's expected suitability for the species could be verified by monitoring results of both surveys. After one year, the Rhine sculpin was detected at every sampling site up to the barrier using eDNA and at every sampling site up to the barrier except for site 1 using electrofishing. Additional sampling following the Rhine sculpin's' next reproduction period is needed to investigate the fish's further dispersal upstream.

#### *Applicability of eDNA for biomonitoring of fish*

Our study shows that eDNA detection can be more sensitive in detecting fish than traditional electrofishing. The electrofishing observations performed here suggest that the Rhine sculpin was not able to cross a minor barrier in the stream. However, the species was detected upstream of the barrier using eDNA, indicating that the mobility of the Rhine sculpin and its capacity to surmount instream obstacles is better than previously predicted. One limitation of our study was that only 20 m of electrofishing was conducted at each sampling site. Given that sampling sites were 200 m apart beyond the barrier, this only covers a short section of stream. However, due to the impracticability of sampling whole streams, many studies use a subsampling approach when conducting electrofishing (Dußling, 2014). It is unrealistic to cover all habitats, especially in wider and deeper streams. Overlooking individuals thus is the rule rather than the exception with electrofishing (Jerde et al., 2011; Pont et al., 2018). Although analysing eDNA to date cannot be

used for estimating individual numbers or age structure, its application in biomonitoring presents a promising, non-invasive and sensitive approach to avoid such damage.

The applicability of eDNA analysis as a monitoring tool is still limited, as many of its characteristics are poorly understood. Though it is known that eDNA release can be affected by size, health, sex or diet of individuals (Goldberg et al., 2015; Klymus, Richter, Chapman, & Paukert, 2015), the extent and variation between species remain unclear. Moreover, eDNA durability in aquatic environments is strongly influenced by degradation through temperature, pH value, conductivity, UV light and microbes (Barnes et al., 2014), reducing eDNA concentration and hence causing additional bias. Furthermore, eDNA movement processes such as downstream transport, soil retention and soil resuspension constantly affect eDNA concentration (Shogren et al., 2017). Apart from these ecological issues, methodological difficulties can lead to failure in detecting eDNA, caused by stochastic effects and polymerase chain reaction (PCR) inhibition (Taberlet et al., 1996; Thomsen & Willerslev, 2015). Together, these may lead to inexplicable patterns of eDNA detection in some studies (*see* Jane et al., 2015; Laramie et al., 2015).

eDNA can be transported long distances downstream in flowing waters (Deiner & Altermatt, 2014; Deutschmann, Müller, Hollert, & Brinkmann, 2019; Jane et al., 2015), which has important implications for this study, as the Rhine sculpin's eDNA can be transported downstream from actual residence sites. This was observed for sample 1 G, where the species was detected even though electrofishing failed to detect individuals up to 20 m upstream of the site. This can lead to the assumed detection of the Rhine sculpin at every site downstream of the species' uppermost actual residence site, representing false positives rather than true local detection. Hence, visual observation of individuals by traditional surveys is still necessary for the validation of local presence at sites downstream of actual residence sites. However, when validating the general presence of a fish species in flowing waters, this effect might be beneficial, as there is no need to sample the whole stream or river.

In this study the Rhine sculpin was not detected at several sites despite the presence of the species further upstream on the same day. These occurrences represent false negatives, which is consistent with comparable studies (Foote et al., 2012; Jane et al., 2015; Laramie et al., 2015) and can be explained by dilution of DNA by high flows, increasing distance from the DNA source or PCR inhibition (Jane et al., 2015) as well as by eDNA movement processes such as retention (Shogren

et al., 2017). Inhibition is especially problematic in eDNA studies, as it can mask even high eDNA copy numbers (Jane et al., 2015) and hence leads to false negatives although the target organism is present (Goldberg et al., 2015; Thomsen & Willerslev, 2015). Addressing PCR inhibition is therefore one of the major challenges in detecting target species, especially in biomonitoring.

### *Conclusion*

Our study shows that eDNA can provide detailed insights into reintroduction success. It allowed for the monitoring of the dispersal of the Rhine sculpin in a small German stream at a fine temporal and spatial scale, revealing a much greater realized dispersal potential than previously assumed and verifying a successful stream restoration. Furthermore, analysing eDNA showed higher detection sensitivity compared to traditional electrofishing in our study. The approach is thus applicable to investigate the ecological status and fragmentation of streams by proving the presence of an indicator species, making it a useful tool for ecological monitoring. Thus, although several aspects need to be further understood to correctly interpret false negative results, eDNA analysis holds great potential for freshwater biomonitoring and is an effective, non-invasive approach that can be used to augment traditional methods.

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# Figures



Fig. 1: (A) Borbecker Mühlenbach, (B) Barrier before site 11, which was assumed to be a dispersal barrier for the Rhine sculpin, (C) Detailed map of the sampling sites at the stream. For coordinates of and distances between sampling sites see Supplementary Data S2.

Date	Day	Sampling event	Sampling site and distance to site 1 in [m]																		B	Sampling site and distance to site 1 in [m]								B
			1	1a	2	3	4	5	6	7	8	9	10		11	11a	11b		12			13	14							
			0	50	100	200	250	300	350	400	450	500	550	600		650	700	750	800	850		1000		1050						
2017-08-15	-8	Neg	0/10			0/10				0/10				0/10				0/10		0/10	0/10									
2017-08-23	0				Reintroduction															0/10	0/10									
2017-08-25	2	A	1/5		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10				0/10												
2017-08-28	5	B	5/5		1/5	1/5	0/10	2/5	0/10	1/10	0/10	1/5	0/10					0/10												
2017-09-02	10	C	1/5	4/5	3/5	4/5	3/5	1/5	1/5	0/10	1/5	0/10	1/10					0/10												
2017-10-06	44	D	2/5	4/5	4/7	5/7	0/10	0/10	0/10	2/7	0/10	0/10	0/10					0/10												
2017-11-02	71	E	2/5	1/5	5/5	3/5	1/5	1/5	1/5	0/10	1/5	0/10	1/5	0/10				0/10												
2017-12-05	104	F	3/5	3/5	4/5	3/5	1/5	0/10	0/10	1/10	0/10	0/10	0/10					0/10												
2018-11-05	439	G	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5					0/10			1/10	0/10								
		E-fishing	0	18	20	5	15	2	10	13	4	18	8					0			0	NA								
2018-12-07	471	H											5/5					5/10	1/10	1/10		0/10	0/10							

Fig. 2: Results of the Rhine sculpin detection by nested end-point PCRs. Positive samples are coloured in dark green, negative samples in orange. Each sample shows the number of positive technical replicates. White text indicates Sanger sequencing for one of the replicates. Electrofishing results are coloured in light blue and show the number of captured individuals per 20 m section.