SHORT COMMUNICATION

Assessing the fate of brown trout (*Salmo trutta*) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe

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Abstract

Environmental DNA (eDNA) analysis in the aquatic environment has emerged as a promising tool for diagnosis of the ecological status in comprehensive monitoring strategies and might become useful in context of the European Water Framework Directive (WFD) and other legislations to derive stressor-specific indicators. Despite many studies having made significant progress for the future use of eDNA in terms of ecosystem composition and detection of invasive/rare species in inland waters, much remains unknown about the transport and fate of eDNA under natural environmental conditions. We designed a specific dual-labelled-probe to detect brown trout (*Salmo trutta*, L.) eDNA and used the probe to describe the fate of eDNA released from an aquaculture facility into the low mountain range stream Wehebach, Germany. The probe was shown to be specific to brown trout, as ponds housing rainbow trout (*Oncorhynchus mykiss*) did not test positive. Even though we observed different strengths of eDNA signals for three ponds containing different brown trout quantities, no significant correlation was found between biomass (kg/L) and eDNA quantity. Our results indicate that the release of DNA from brown trout might be life stage and/or age-dependent. The effluents of the aquaculture facility were a source of high levels of eDNA which resulted in the greatest abundance of brown trout eDNA directly downstream of the facility. Despite the natural occurrence of brown trout in the Wehebach, as shown by ecological investigations conducted by authorities of the federal state of North Rhine-Westphalia (Germany) and personal observations, we observed a significant decrease of relative abundance of eDNA in the Wehebach within the first 1.5 km downstream of the aquaculture. Our results suggest that concentrations of eDNA in running waters rapidly decrease under natural conditions due to dilution and degradation processes, which might have important implications for the utility of eDNA in environmental research.

Keywords: eDNA, fate, brown trout, rainbow trout, aquaculture, qPCR

Introduction

Since the term ’environmental DNA‘ (eDNA) was first introduced by Ogram et al. (1987) in the field of microbiology, eDNA analysis has been evolving rapidly into a powerful monitoring tool for the assessment of aquatic species distributions (Baldigo et al., 2017). Biodiversity of aquatic systems is a key element of ecological function (Risser, 1995) and species losses at different scales can significantly impact the health of ecosystems (Beermann et al., 2018; Valentini et al., 2016). Therefore, species richness is a fundamental criterion in most ecological concepts and models for the assessment of environmental quality (Gotelli and Colwell, 2011). Estimating population abundances is a traditional and common objective in fisheries management and conservation, but classical methods (e.g. electrofishing, visual observations, gillnets) are challenging because of the associated costs and logistic, and they might harm or miss target organisms (Lacoursiere-Roussel et al., 2016; Doi et al., 2016). This is particularly problematic since uncommon, rare, or elusive species are critically important for accurate estimations of the species richness of aquatic ecosystems (Doi et al., 2016; Olds et al., 2016). As a potential alternative, eDNA methods can be a valuable tool for detecting many species that are difficult to study by traditional methods (Rees et al., 2014). Nonetheless, it is important to point out that the study of eDNA is not necessarily comprehensive either, as previous research has failed to detect the target species where it was known to occur (Thomsen et al., 2012b). Often, however, eDNA analysis has been successfully applied as a monitoring tool for the estimation of presence/absence of even rare and/or invasive species and can be used for ecological quality assessment (Furlan and Gleeson, 2016; Lacoursiere-Roussel et al., 2016). By using this technique, there is a realistic potential for more complete species lists in support of ecological assessment and management of aquatic ecosystems (Olds et al., 2016), and a significant reduction of costs associated with data collection and negative impacts on studied organisms (Lodge et al., 2012; Taberlet et al., 2012). Analysis of eDNA would be a particularly effective tool for large-scale monitoring campaigns (Doi et al., 2016). Novel tools for diagnosis of the ecological status should be used for comprehensive monitoring strategies and the revision of the Water Framework Directive (WFD) including stressor-specific indicators such as

72 -omics approaches, next-generation sequencing of organisms exposed or collected in the field
73 (Brinkmann et al., 2016; Carusi et al., 2018) and eDNA metabarcoding (Xie et al., 2018) may enhance
74 the throughput of structure-based assessment of ecosystems and provide more direct links between
75 chemicals and their modes of action (MoA) and ecosystem functions (Brack et al., 2018; Pawlowski et
76 al., 2018).

77 Analysis of eDNA is often based on non-invasive extraction of bulk environmental samples (e.g. soil,
78 water, air) without obvious signs of the presence of the biological source organism (Taberlet et al.,
79 2012; Thomsen and Willerslev, 2015; Furlan and Gleeson, 2016). The genetic materials may be
80 composed of extracellular and/or DNA within cells or organelles derived from skin, gills, urine, feces,
81 mucus or gametes (Taberlet et al., 2012; Lacoursiere-Roussel et al., 2016). Typically, mitochondrial
82 DNA is targeted because of its abundance in cells and effectiveness in identifying organisms to the
83 species level (Hebert et al., 2003). Nevertheless, nuclear DNA markers can be used and have the
84 potential to address issues that could not be addressed by using mitochondrial DNA markers, such as
85 the estimation of population genetic diversity and introgression levels in invaded populations
86 (Minamoto et al., 2016). Studies in terms of presence/absence of species have been successfully
87 performed using quantitative real-time PCR (qPCR) with species-specific primers (Takahara et al., 2012;
88 Pilliod et al., 2013; Ikeda et al., 2016; Yamanaka and Minamoto, 2016). Metabarcoding using universal
89 primers and high-throughput next-generation sequencing of eDNA is another approach to assess
90 aquatic community structures (Thomsen et al., 2012a; Miya et al., 2015; Yamamoto et al., 2017). In
91 addition to presence/absence analyses, several studies revealed positive correlations between
92 abundance/biomass of an organisms and abundance of eDNA in laboratory tanks or in lakes and ponds;
93 to a lesser extent, such correlations have been described in streams and rivers (for review: Doi et al.,
94 2016) which are of importance for freshwater ecology as well as freshwater fisheries (Matthews, 1998;
95 Doi et al., 2016).

96 Quantitative estimation of population size in streams is challenging because the persistence and
97 dispersion of eDNA due to hydrological processes are largely unknown and have received little

attention (Doi et al., 2016; Lacoursiere-Roussel et al., 2016; Baldigo et al., 2017). Notwithstanding, our knowledge of environmental factors that affect the fate of eDNA in the environment is still far from complete but crucial for standardized sampling and analysis procedures (Baldigo et al., 2017). The abundance of eDNA in water samples reflects the quotient of the rates of eDNA release and degradation, both of which may strongly be altered by environmental conditions (Lacoursiers et al., 2016), such as microbiological activity, seasonal conditions, nature of eDNA, ultraviolet radiation, hydromorphological conditions, temperature and behavior of fish (Zhu, 2006; Pilliod et al., 2013b; Lacrousiere-Roussel et al., 2016; Song et al., 2017). DNA fragments are suggested to persist for up to few weeks in freshwater ecosystems (Lacoursiere-Roussel et al., 2016). Downstream transport of eDNA up to 50 km has been reported in flowing waters under natural conditions (Deiner and Altermatt, 2014). As suggested by Takahara et al. (2012), greater temperatures lead to increased degradation of DNA molecules due to greater enzyme activities and microbial metabolism. On the other hand, elevated temperatures have significant effects on fish (growth, metabolism, physiology, immune function) and therefore might increase eDNA release (Takahara et al., 2012).

The goal of our study was to estimate the transport distance of eDNA of brown trout (*Salmo trutta*, L.) released from aquaculture ponds of a local aquaculture in Stolberg, Germany. We designed species-specific primers and a dual-labelled probe for the detection of brown trout eDNA and used qPCR for relative quantification in water samples. Sampling sites included various locations within the aquaculture facility, and in the low mountain range stream Wehebach upstream and downstream of the outfall of the facility. The Wehebach flows in northern direction through the federal state of North Rhine-Westphalia and is dammed (reservoir 'Wehebachtalsperre') before it flows into the river Inde. To demonstrate species-specificity, samples from ponds containing rainbow trout (*Oncorhynchus mykiss*) were also analyzed. Due to the fish monitoring program of the Water Framework Directive (WFD; Directive 2000/60/EC) and personal observations, a natural occurrence of brown trout in the Wehebach upstream and downstream of the aquaculture was confirmed.

Methods

Study concept

Environmental DNA was sampled in the low mountain range stream Wehebach and an adjacent facility for aquaculture of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) in Stolberg, Germany, on July 6, 2016 per previously described protocols (Laramie et al. 2015). The conceptual flow diagram of the fish aquaculture facility 'Mohnen' (Stolberg, Germany) is shown in Figure 1A. Locations of sampling sites along the Wehebach are illustrated in Figure 2A.

The different ponds contained groups of fish of different species, biomass, number of individuals, water volume and life stage. Water is supplied to the facility from the Wehebach. Water was aerated before the flow was split into several initial parallel ponds, from where it entered a series of downstream ponds. Before being discharged into the Wehebach, a treatment process was used to remove particulates and organic matter from the outflow. The volume flow from the aquaculture facility into the Wehebach amounted to approx. 50 L s^{-1}.

Samples for eDNA analysis were collected upstream of the aquaculture facility in the Wehebach, the inflow of pond 1, the outflow of pond 1 and downstream of the outflow of pond 1 in the Wehebach (but upstream the post-treatment outflow of the rest of the facility). Additionally, samples were taken from the collective inflow of ponds 2-4, the individual outflows of pond 2, 3, and 4, as well as untreated collective outflow and treated collective outflow of the facility.

To analyze the transport and fate of eDNA discharged from the aquaculture facility, sampling sites 1.5, 2.0, 4.0, and 6.0 km downstream along the Wehebach stream were analyzed. Figure 2 shows the different sampling sites and their distance from the facility.

144 **Sampling of environmental DNA**

145 Briefly, grab samples of surface water were carefully taken and collected in autoclaved 1 L
146 polypropylene bottles (Nalgene, Fisher Scientific, Schwerte, Germany). Surface water sampling was
147 appropriate as water in all studied matrices was well-mixed. Samples were immediately refrigerated
148 and transported to the laboratory, where eDNA was extracted on the same day. To this end, 1 L of
149 water were vacuum-filtered through sterile disposable funnel units with pre-assembled 0.45 µm
150 nitrocellulose filters (47 mm diameter; Nalgene, Fisher Scientific). Filters were folded using
151 decontaminated forceps (70% ethanol for 10 min, 10% bleach for 10 min, rinsed with RNase AWAY
152 decontamination reagent, Fisher Scientific) and stored in cryogenic tubes containing ultrapure ethanol
153 (Sigma-Aldrich, Steinheim, Germany). Each filter was shredded, and total DNA extracted using the
154 Qiagen DNeasy Blood and Tissue kit as per the manufacturer's protocols (Qiagen, Hilden, Germany).
155 Total DNA concentration in the extracts was determined by use of a BioDrop µLITE Spectrophotometer
156 (BioDrop, Cambridge, UK) and the samples stored at -20 °C until further analysis.

157 For quality control and to identify potential cross contamination, a field blank was established. For the
158 field blank, ultrapure water was filled in sampling bottles and bottles were opened at each sampling
159 site and analyzed in the laboratory like the collected samples.

160

161 **Design of primers and dual-labelled probe**

162 Species-specific primers and a dual-labelled probe for the cytochrome c oxidase subunit 1 (*cox1*) gene
163 of brown trout (*Salmo trutta*) were designed based on the whole mitochondrial genome sequence
164 information from NCBI gene bank, accession number NC_024032.1 (5475..7025). The *cox1* is gene is
165 particularly well-suited for eDNA analysis since as a mitochondrial gene, it is highly abundant in the cell
166 and has been characterized in many species, thus facilitating the development of specific primers and
167 probes. Oligonucleotides were designed and ordered through the Eurofins Genomics (Ebersberg,

168 Germany) qPCR primer and probe design tool (Table 1). The qPCR probe was 5'-labelled with 6-FAM
169 (Fluorescein) and 3'-modified with TAMRA™ (TAM).

170 The efficiency of this primer-probe combination was determined using 10-fold serial dilutions of a
171 composite sample of total DNA extracted from the livers of four brown trout specimens as described
172 above, with a total DNA concentration of 12.6 ng μL^{-1} (Figure 2B).

173

174 **Quantitative real-time RT-PCR (qPCR)**

175 The abundance of brown trout *cox1* DNA in eDNA samples was quantified in duplicate by means of
176 quantitative real-time PCR (qPCR) using a 96-well StepOne Plus real-time PCR system (Applied
177 Biosystems, Foster City, CA, USA) according to previously published methods (Gustavson et al. 2015).
178 Briefly, 30 µL duplicate reactions were set up that contained 15 µL TaqMan master mix with ROX as
179 the passive reference dye (Applied Biosystems), 3 µL of each forward and reverse gene-specific primers
180 (2 µM each), the dual-labelled probe (2 µM), as well as 3 µL DNase/RNase-free water (Fisher Scientific)
181 and 3 µL template. The qPCR was run at 50 °C for 5 min and 95 °C for 10 min, followed by 40 PCR cycles
182 (95 °C for 15 s denaturation, and 60 °C for 1 min annealing and extension).

183

184 **Fish data**

185 Data of fish abundance in the Wehebach were obtained from the database 'Fischinfo Nordrhein-
186 Westfalen' (database for the acquisition, evaluation and management of fish data) published by the
187 State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia, Germany.
188 Fish data were collected within the Water Framework Directive (WFD) monitoring program. Fish were
189 caught *via* electrofishing. WFD sampling sites were comparable to eDNA sampling sites 1.5 km, 4.0 km
190 and 6.0 km downstream of the facility.

Data analysis and statistics

Abundance of brown trout DNA equivalents in eDNA samples was interpolated using the relative standard curve method, normalized to DNA yield during extraction of samples and the volume of water extracted, and expressed as ng brown trout DNA equivalents per L of water. Data were normalized to values measured in water from upstream of the facility (samples from in- and outflows of the ponds) or directly downstream of the outflow of the treated effluent (longitudinal stream samples) and expressed as x-fold differences. Values obtained from the various sampling sites were analyzed for statistical differences by means of One-way ANOVA with Tukey's post-hoc test ($p \leq 0.05$) using Prism 7 software (GraphPad, LaJolla, USA).

Results and discussion

Species-specificity of the designed primers was confirmed by eDNA results obtained from water samples from different ponds at the aquaculture facility (Figure 1C). Whereas water samples from the outflow of the pond 3 populated with adult brown trout showed very high values for the relative brown trout eDNA concentration (1,910-fold greater compared to water upstream of the facility), the outflow of a pond populated with rainbow trout showed a value that was 0.41-fold of that in upstream water. Effectively, the passage through the rainbow trout pond decreased the brown trout eDNA signal from the inflow (1.3-fold that of upstream water). Additionally, brown trout eDNA concentration in water from the outflow of pond 1 (Figure 1B), populated with adult brown trout, was 42.6-fold greater compared to the inflow. Of all studied ponds, the outflow of that containing brown trout brood showed the lowest value (2.8-fold compared to upstream water). Our field blank tested negative for brown trout eDNA; therefore, it can be assumed that no cross-contamination occurred either during field-sampling or the subsequent laboratory processes (Figure 1B).

eDNA and fish biomass

In the last years several studies have attempted to estimate the biomass of aquatic organisms from the abundance of eDNA for conservation purposes. Since biomass is a fundamental biological parameter, eDNA seems to be a promising tool for this purpose since data from traditional techniques (electrofishing, net fishing or hydroacoustic) might not reflect the actual population structure and biomasses adequately. Most of these studies were performed in standing waters or aquariums (Thomsen et al., 2012a; Takahara et al., 2012; Pilliod et al., 2013). They found a quantitative linear relationship between the number of eDNA molecules in the water and biomass (biomass/L) of the target organisms. However, in our study we did not observe such a direct relationship (Table 1).

In the present study, only three ponds with known biomass were available for analysis of quantitative relationships between biomass and abundance of eDNA. No linear relationship as found in previous reports could be established in the present study. The biomass of brown trout brood (pond 4) was comparable to that of pond 3 containing adult brown trout, but the eDNA signal was considerably lower. One factor which might have influenced this result was a greater exchange range of fresh water in the much smaller pond containing the fish breed, which consequently resulted in a lower abundance of eDNA. The volume of fresh water flowing into ponds 3 and 4 was equal, while pond 3 is approx. 12-times greater in volume compared to pond 4.

Another factor which is often underrated in eDNA studies and determines the amount of eDNA released from an organism is the life stage. Species interact with their environment and continuously release eDNA to their surroundings *via* shedding of cells, urine, feces, hair/skin, and gametes, as well as from dead individuals leaking genetic material (for review, see Thomsen and Willerslev, 2015). In the case of fish, expulsion of DNA from the mucous layer is an important factor. Considering the different sources of fish DNA in the water phase it is very likely that different life stages and/or sizes of individuals might influence the amount of released DNA and should be considered if eDNA analysis is used for biomass estimation. This aspect should be the subject of dedicated future research.

Transport and fate of eDNA

In our study we were able to demonstrate that presence of brown trout in pond 1 (Figure 1B) resulted in a significant increase in relative brown trout eDNA in the outflow (0.28-fold that of upstream water at the inflow of pond 1 compared to 42.6-fold at its outflow). A comparable eDNA signal was observable directly downstream of the aquaculture facility (38.4-fold compared to that of upstream water) where all effluents are collectively discharged into the stream. Significantly elevated relative eDNA concentrations where also demonstrated for pond 3 (1,910-fold compared to that of upstream water, Figure 1C). This was also the greatest eDNA concentration measured in the present study. We furthermore observed a decrease in relative eDNA concentration in the collective untreated outflow (6.88-fold compared to that of upstream water), which is likely the result of dilution of the strong signal from pond 3 with water from pond 2 and 4, which showed relative low eDNA concentrations (pond 2, rainbow trout: 0.41-fold compared to upstream water; pond 4, brown trout brood: 2.82-fold compared to upstream water). After being treated in the treatment process of the aquaculture facility, the relative eDNA concentration was reduced by more than 50% compared to the untreated collective outflow which may indicate a reduction in DNA due to the treatment process, most likely because of the removal of particulate matter.

Downstream of the aquaculture (0 km/downstream; 38.4-fold compared to upstream water; Figure 2C) we observed greater values of relative brown trout eDNA concentration compared to the treated outflow of the aquaculture facility (2.90-fold compared to upstream water). Results suggest that the origin of brown trout eDNA in the Wehebach downstream of the aquaculture were dominated by the effluents of pond 1 (Figure 1B), which receives less rigorous treatment compared to the effluent of the rest of the facility. Table 2 lists the abundance of brown trout in the Wehebach obtained from the fish information platform of the authorities (LANUV) in North Rhine-Westphalia, Germany.

Transport and fate of eDNA are challenging to assess and directly correlated with the degradation pressure on the DNA molecule. The term “state” describes the different physical forms of the eDNA

molecule, such as extracellular, intracellular, free dissolved or particle-bound (Pietramellara et al., 2009; Levy-Booth et al., 2007; Nielsen et al., 2007; Barnes et al., 2014; Turner et al., 2014). Bound to particles, e.g. organic matter, DNA molecules are much more resistant to degradation (Turner et al., 2015). Turner et al. (2015) demonstrated, that eDNA is more concentrated in surficial sediments than in surface water and therefore recommend taking sediment eDNA analysis into account when planning a sampling campaign. Additionally, it was proven that eDNA in sediments is stable and detectable for longer periods of time compared to the water phase. The rapidly progressing field of sediment eDNA analysis provides excellent complementary tools for ecological management and great opportunities to determine historical patterns of community composition. Nevertheless, using sediments for presence/absence studies can lead to an overestimation of the results since released extra-membranous DNA of an organism may persist over a long time and fish might have already migrated or died.

Dissolved in the water phase, several factors promote rapid degradation of DNA which generally follows an exponential decay pattern and therefore depends on the starting concentrations of DNA in its medium (Barnes et al., 2014; Thomsen et al., 2012a; Turner et al., 2015). First, degradation is positively correlated with the length of the DNA molecule (Dejean et al., 2011; Rees et al., 2014). Therefore, it is recommended to use small DNA fragments within the range of 90-120 bp for species detection and mitochondrial DNA markers (as in the present study) to reach higher copy numbers (Rees et al., 2014). Eukaryotic cells can contain up to several hundred mitochondria, but only one nucleus. Thus, choosing mitochondrial genes for eDNA analyses provides greater abundances of total DNA yield. Second, several abiotic and biotic drivers of DNA degradation affect the lifetime of an eDNA signal, namely hydrology, temperature, pH, conductivity, microbial community composition, biological oxygen demand, chlorophyll a concentration, and UV radiation (Barnes et al., 2014). Barnes and his group highlighted in their literature review the importance of the activity of microbes and their enzymes (both intra- and extracellular). The governmental abundance data underline that native

specimens of brown trout were likely present in the entire eDNA sampling stretch along the Wehebach and therefore affected the baseline brown trout eDNA concentrations obtained in this study.

The relative eDNA concentration in the Wehebach decreased significantly within the first 1.5 km from 38.4-fold to 26.3-fold compared to that upstream of the facility. Additionally, all sites downstream of the aquaculture showed significantly lower values compared to the outflow of the aquaculture (2.0 km: 15.4; 4.0 km: 3.62; 5.0 km: 9.47). The results highlight that the eDNA signal rapidly declined with the distance from the aquaculture. Our results are in accordance with literature data. Jane et al. (2015) caged trout in two different fishless streams and their results suggest that a general interaction occurred between flow and distance from the source. For that reason, it can be assumed that eDNA concentrations in flowing waters do not only depend on the equilibrium between DNA released into the water and its degradation. Deiner and Altermatt (2014) used DNA from mussels (*Unio tumidus*) and gastropods (*Daphnia longispina*) to estimate the transport distance of eDNA from a lake where these organisms occur, but not in the receiving stream. For both species, there was an overall significant decrease in detectability of eDNA with increasing distance (up to 13 km downstream from the lake). Additionally, the authors used a generalized linear model (GLM) to predict detection rates beyond their studied distances. They found that the detection threshold of *Unio tumidus* falls below 5% at about 15 km and 25 km in fall and summer, respectively, and for *Daphnia longispina* within about 50 km. Abundance of eDNA differed for both species, whereas the values for the gastropod were greater compared to the mussel. As a potential explanation, the authors suggest different rates of DNA release or downstream drift of *Daphnia longispina* in the stream.

Interestingly, at the sampling site 5.0 km downstream of the aquaculture facility, we observed an increase in relative eDNA abundance compared to the sampling site 4.0 km downstream of the facility. These data suggest that between both sampling sites (or directly at the 5.0-km sampling site) a source of brown trout was present. Two tributaries flow into the Wehebach at the site 5.0 km downstream of the facility. In addition, the Wehebach appeared to be receiving waters from a pond that had traditionally been used for keeping trout, and where residents and visitors to date still frequently feed

fish. Additionally, fish survey data (extrapolated to 300 m data) indicate a high abundance of the local brown trout population at this sampling site, which may be related to the confluence with the two tributaries.

Conclusion

In our study we were able to demonstrate that the designed probe and primers were species-specific for brown trout and can be used to accurately detect the presence of brown trout in ponds and stream water. Under natural environmental conditions (during mid-summer) we observed a rapid and steady decrease of brown trout eDNA concentration downstream of the main source, a local aquaculture facility. The factors driving this decrease are expected to be manifold. An exponential decay of eDNA in aquatic systems has been reported previously. Biomass of fish appeared not to be the only parameter governing the rate of DNA release, and the life stage and age of fish might be a potential factor to be considered. These factors should be the subject of future dedicated research and are beyond the scope of the present study.

In conclusion, eDNA analysis is a powerful tool for future population investigations and ecological assessments. The greatest advantage is its use as an ’early-warning system’ for invasive or endangered species. Additionally, a possible application of eDNA studies for environmental assessment unexhausted by far is the early detection of parasites which may affect the fitness and survival of entire populations. Given the knowledge gaps that are still apparent regarding the lifetime of eDNA signals and the factors influencing them, eDNA studies should be considered complementary rather than ’stand-alone tools‘, particularly in flowing waters.

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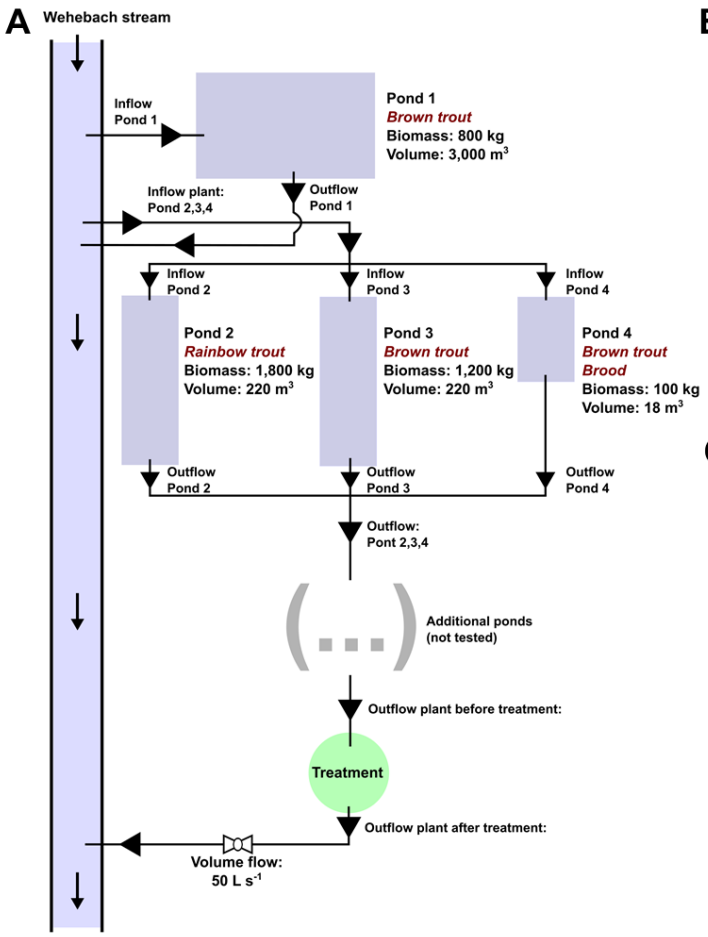
Figures



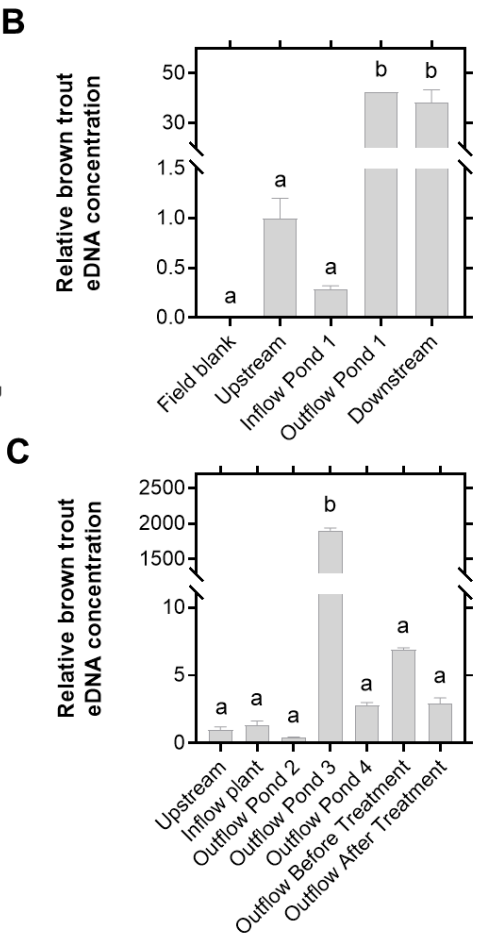
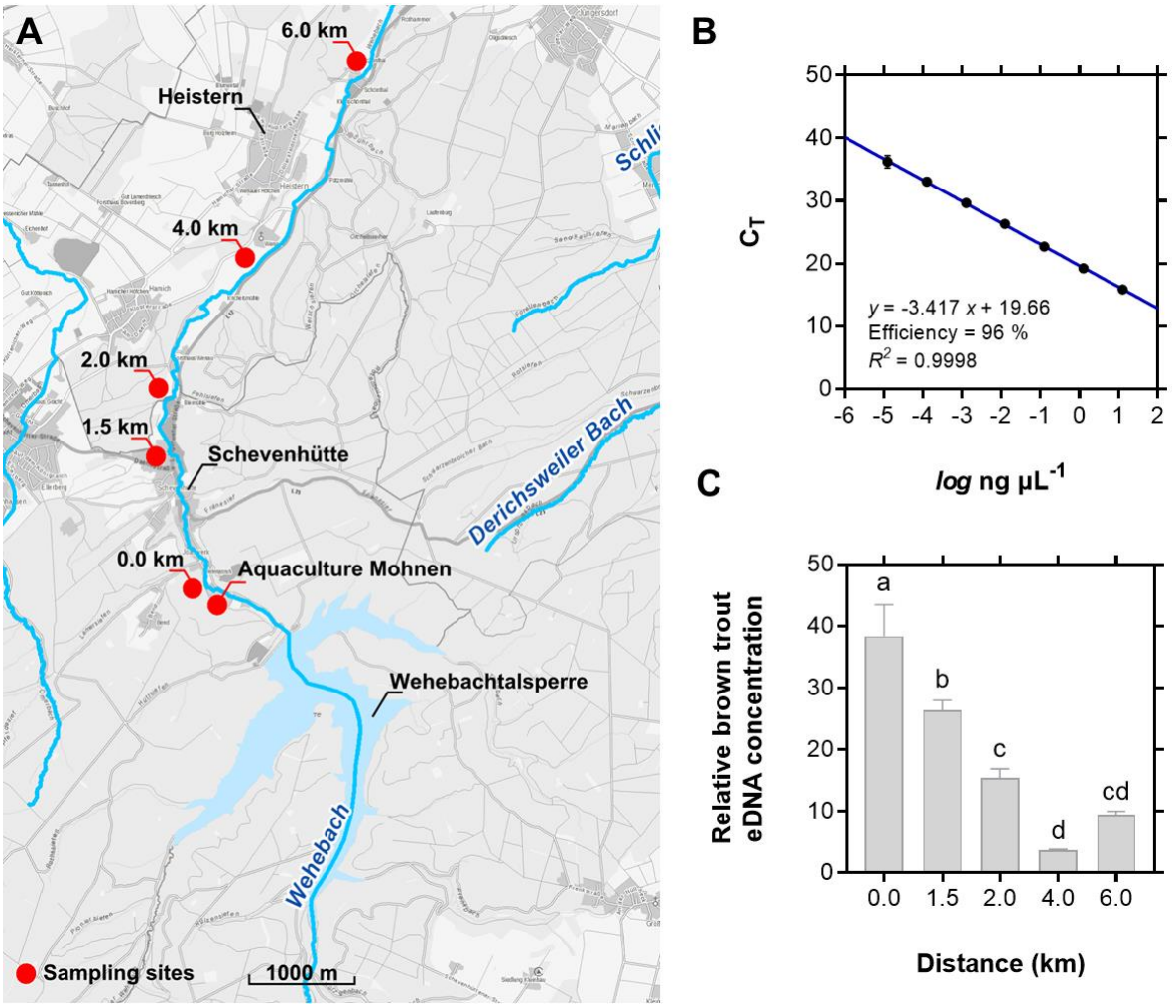


Figure 1. (A) Conceptual flow diagram of the studied aquaculture facility in Stolberg, Germany. (B) Brown trout eDNA concentrations of the field blank, pond 1 of the aquaculture and down/upstream of the aquaculture facility in the Wehebach relative to the upstream sampling location. (C) Brown trout eDNA concentration at different sampling sites on the aquaculture facility relative to that in the upstream sampling location. (B/C) Bars represent the mean value of relative eDNA concentrations. Error bars represent the standard deviation. Bars sharing the same letter are not significant different from one another (One-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$).



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465 **Figure 2.** (A) Sampling sites for eDNA along the Wehebach stream in Stolberg, Germany. (B) Linear
466 regression of the cycle threshold (CT) in qPCR reactions with a dual-labelled eDNA probe for brown
467 trout cytochrome c oxidase subunit I (*cox1*) against concentration of the mixed eDNA standard. Circles
468 and error bars represent mean values and standard deviations of two duplicate determinations.
469 Amplification efficiency, coefficient of determination (R^2) and the regression line are provided in the
470 legend. (C) Relative brown trout eDNA at different locations along the Wehebach. Bars represent the
471 mean value of relative eDNA concentrations. Error bars represent the standard deviation. Bars sharing
472 the same letter are not significant different from one another (One-way ANOVA with Tukey's post-hoc
473 test, $p \leq 0.05$).

Table 1: Biomass (kg/m^3) and concentration of relative brown trout DNA of different ponds of the aquaculture.

	Pond 1 (adult brown trout)	Pond 3 (adult brown trout)	Pond 4 (brown trout brood)
Biomass (kg/m^3)	0.27	5.45	5.56
Relative brown trout eDNA concentration	42.6	1,910	2.82

Table 2: Abundance of brown trout determined *via* electrofishing in the Wehebach obtained from different monitoring studies. Data obtained from Fischinfo database: http://fischinfo.naturschutzinformationen.nrw.de/fischinfo/de/start.

Sampling site	Number of brown trout	Length fishing stripe	Reference
Upstream aquaculture	83	150 m	Fish ecology investigation 13.07.1999
	191	400 m	Fish ecology investigation 19.12.2006
200 m downstream of the aquaculture	337	150 m	Fish ecology investigation 13.07.1999
4 km downstream	99	300 m	WFD Monitoring 02.09.2010
	94	300 m	WFD Monitoring 12.09.2013
	155	300 m	WFD Monitoring 09.09.2016
6 km downstream	69 (207)	100 m (300 m)	Fish ecology investigation 20.09.1996
	80 (240)	100 m (300 m)	Fish ecology investigation 17.09.2005
	46 (138)	100 m (300 m)	Fish ecology investigation 10.09.2006