# Comparing qPCR and eDNA-metabarcoding for detection of elusive lampreys

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# Abstract

With the world potentially approaching the sixth mass extinction, it is imperative that species we lack knowledge about are investigated to learn more about their distribution. An efficient way to acquire knowledge about many threatened and elusive freshwater fish species is through environmental DNA monitoring. eDNA monitoring has begun to be a staple surveying tool to investigate distribution of different species due to it being cost- and time-effective, and reliable. There are different methods within eDNA monitoring, such as qPCR and DNA-metabarcoding. qPCR is a species-specific real-time quantitative analysis method, where species are investigated one at a time, whereas DNA-metabarcoding investigate multiple freshwater fish species at once.

In this study I tested and compared qPCR and DNA-metabarcoding methods for studying the distribution of river (*Lampetra fluviatilis*) and brook (*Lampetra planeri*) lamprey, in a river system where they are known to reside, Haldenvassdraget in the south-east of Norway. Currently it is impossible to distinguish river and brook lamprey genetically, so in this study they are treated as one species. I collected two independent water samples from 45 locations in Haldenvassdraget in an attempt to cover the whole river system. From the 45 locations 21 showed presence of lamprey, and 24 did not. Lamprey were primarily found in the upper parts of the river system and two rivers in the lower parts of the system, while no lamprey were found in the tributary Setten. In total, DNA-metabarcoding analyses on 16 samples failed, as such there is a possibility that the corresponding qPCR analyses on the same 16 samples also failed. Two generalised linear mixed model analyses were performed to test if the methods, qPCR and DNA-metabarcoding, differed in detection probability of lamprey. Sampling locations were added as a random factor. Water volume and temperature were added as fixed factors. The first analysis was done on the data set including results from both methods, excluding the 16 samples from DNA-metabarcoding that did not work as intended. The second analysis was done on the same data set where the 16 samples qPCR samples corresponding to the failed DNA-metabarcoding samples were removed. For the first analysis, detection probability did not differ between methods, but in the second analysis qPCR was shown to have a statistically significant higher detection probability than DNA-metabarcoding. Unexpectedly, for both analyses water volume had a negative impact on detection probability.

This study supports prior literature that qPCR in general is a more reliable method. I would also say that the study supports DNA-metabarcoding as a reliable alternative to qPCR when the objective is to investigate diversity and distribution for multiple freshwater fish. Also, in the case of lamprey, a species-specific protocol, where their life history and habitat preferences are taken into account, could be beneficial.

# Acknowledgments

I had great hopes for my time as a master's student, but alas, with a pandemic and my health issues working against me, my grand plan did not come to fruition. Nevertheless, here we are, a master thesis delivered and one more student ready to join the grownups at work.

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# Contents

# 1 Introduction

## 1.1 Monitoring freshwater fish distribution

Sustaining biodiversity is becoming increasingly important to avert the Earth's trajectory towards the Sixth Mass Extinction (Cowie et al., 2022). Biodiversity is in danger of degradation at the genetic, species, and ecosystem level (Wrona et al., 2006), and 50% of all freshwater fish species are at threat from climate change worldwide (Ahmed et al., 2022). Monitoring must therefore take place to improve and ensure biodiversity. Environmental DNA (eDNA) monitoring is increasingly more popular as a tool to investigate biodiversity. Not only to find invasive (Fossøy et al., 2018) or endangered fish species (Thomsen et al., 2012), but also as a tool to investigate the distribution of local species (Rees et al., 2014; Rourke et al., 2022). eDNA offers a more objective method of monitoring as it does not rely on traditional taxonomic expertise in the field, long-term effort and does not disturb species being investigated (Fossøy et al., 2018; Strand et al., 2019). The method is based on that all organisms release DNA into their surroundings, and that it can be collected, for example by filtering water. DNA breaks down rapidly in nature. It is greatly influenced by temperature and water quality (Eichmiller et al., 2016), so a finding of one or more species indicates a high probability that they are present or have been in the area within a relatively short period of time (Balasingham et al., 2017). eDNA can therefore reflect the temporal and local diversity (Rees et al., 2014). The detection range of eDNA is disputed. In smaller rivers eDNA had a detection range of less than one km (Jane et al., 2015; Wilcox et al., 2016). In the outlet of lakes, dwelling species were detectable up to 9.1 km (Deiner & Altermatt, 2014). The further away from where the eDNA originated, the lower the probability of successful detection. eDNA can, however, potentially be detected at distances of up to 130 km (Pont, D. et al. 2018).

Compared to traditional monitoring methods eDNA methods are more sensitive and finds more species (Thomsen et al., 2012; Valentini et al., 2016), while also being less costly and time consuming. A study from 2018 investigating the downstream transportation ability of eDNA in rivers found that their sampling campaign of 12 days was equivalent to ten years of traditional sampling effort in order to reach an accurate image of fish biodiversity in the area (Pont et al., 2018).

In this study two eDNA methods were compared, qPCR and DNA-metabarcoding. Although there is comparable similarity between the methods (Yu et al., 2022), they are different in several ways. qPCR uses species-specific genetic markers to identify specific species, whereas DNA-metabarcoding uses a universal PCR primer pair to identify many species withing a taxonomical group, such as freshwater fish (Miya et al., 2020). qPCR as a monitoring method is more established than DNA-metabarcoding and perceived as more reliable (Bylemans et al., 2019; Lecaudey et al., 2019; Schenekar et al., 2020). Nevertheless, DNA-metabarcoding is seen as more suited for monitoring distribution of diversity in large river systems (Harper et al., 2018; Lecaudey et al., 2019).

As with all monitoring methods there are some drawbacks. eDNA methods are dependent on PCR where inhibiting substances in the water sample may affect the detection ability and potentially lead to false-negative results (Jane et al., 2015; Schrader et al., 2012). A known downside of DNA-metabarcoding is amplification bias (Bylemans et al., 2019; Elbrecht & Leese, 2015; Kelly et al., 2019; Tremblay et al., 2015). The MiFish primers are intended to cover a wide range of freshwater fish species (Miya et al., 2015). If there are species with relatively high abundance in the sample there is a chance that this abundance will block the detection of species with low DNA abundance. This amplification can also lead non-target-species DNA to be amplified (Alberdi et al., 2018; Gargan et al., 2022; Nichols et al., 2018; Piñol et al., 2019). For MiFish there might be difficulties with primer/template mismatches leading to underrepresentation of known species (Miya et al., 2020). Another downside to DNA-metabarcoding is the strong dependency on the comparison database; if a species DNA reference is missing then no match can occur. Moreover, there are also multiple families where it is not possible for DNA-metabarcoding to distinguish between individual species within the family (http://mitofish.aori.u-tokyo.ac.jp/species/all/).

## 1.2 Little-studied fish in Norway: Lamprey

In this study, I will be investigating two species that are genetically indistinguishable (Docker, 2014; Zancolli et al., 2018) and are grouped as a paired species, river lamprey (*Lampetra fluviatilis*) and brook lamprey (*Lampetra planeri*). In this pair the river lamprey is the parasitic ancestor to the non-parasitic derivative brook lamprey (Docker, 2014; Zancolli et al., 2018). In Norway, river lamprey and brook lamprey are regarded as two different species, due to their clear morphological differences in their adult phase and differences in life cycle (Pethon, 2005; Spikkeland & Kasbo, 2014). Both lamprey species are known in Norway along with two other species which are not a part of this study, sea lamprey (*Petromyzon marinus*) and Arctic lamprey (*Lethenteron camtschaticum*). Lamprey distribution has not been sufficiently investigated in Norway (Hesthagen et al., 2021; Schartum & Kraabøl, 2013), and knowledge of lamprey distribution comes mainly from general fish diversity studies and bycatch data. In Haldenvassdraget, a river system located in eastern Norway, interest in lamprey species native to the region have begun to increase. A 2014 review about the distribution of river and brook lamprey in Haldenvassdraget reported that river lamprey was observed in, from north to south, Haretonelva (Ulviksjøen), Maltjennbekken, Langfossjøbekken (Longselva), Ørje, Fisma by Femsjøen (Rødselva). One brook lamprey was observed in Ørje, and unspecified lamprey species were observed in Midtskogvassdraget, Hemnessjøen, and Hafsteinselva (Spikkeland & Kasbo, 2014). A comprehensive review from 2021 continued to build on the knowledge of brook lamprey in Haldenvassdraget. Brook lamprey was observed in Bjørkelangen, Komnesbekken, Prestelva/Riselva, Gorobekken, Taraldrudelva (three observations), Rødenessjøen, Buerelva (three observations), Store Le, and Mellebyelva (Hesthagen et al., 2021). In addition to reviews three observations of river lamprey have been registered in Artsdatabanken (Artskart Artsdatabanken), which was not included in the reviews, were Mjerma, Femsjøen (Huitfeldt-Kaas, 1918), and Tista where it meets Iddefjorden. With their presence confirmed in Haldenvassdraget, it is reasonable to expect that I will be able to identify more locations where river and brook lamprey reside.

Figure 1 Top: river lamprey, bottom: brook lamprey. Owned by Jan Fekjan, license CC BY-SA 4.0

River and brook lamprey are suitable for this eDNA study because they represent species that are elusive and rare, resulting in them not being easily detected by traditional methods (Hesthagen et al., 2021; Huitfeldt-Kaas, 1918; Spikkeland & Kasbo, 2014). Based on their life cycle and habitat preferences it is probable that they appear throughout the river system even in places they have yet to be observed. Their life cycle and life history strategies are not well studied in Norway. Findings from a brook lamprey study from Telemark found that the life cycle and life history of brook lamprey in Norway follows the established knowledge of brook lamprey globally. I will therefore assume that any findings of brook lamprey globally will also hold true for brook lamprey in Haldenvassdraget, unless there is clear evidence to the contrary. There is a lot of research being done on lamprey on a world basis, and Docker (2014) has summarised and compiled the most comprehensive review of lamprey biology as of today, in the book *Lampreys: Biology, Conservation and Control, Volume1*. From this book I have summarised relevant knowledge for this study. Both species begin their life cycle in freshwater, with a protracted larval phase, typically lasting between 3-7 years. During this phase they burrow down into soft sediment, which gives them protection from predators and allows them to filter feed on organic matter. When burrowed the larvae are difficult to observe and even more difficult to identify, leading to lampreys rarely being registered. Due to their worm-like shape, they are often mistaken as worms. They reside in streams and rivers, where the water is slow flowing and highly oxygenated. The larvae disperse largely based on factors such as changes in water velocity and water levels. However, they have some form of locomotion and actively seek better habitats for colonization. Important to note is that their ultimate upper incipient temperature is 30 °C, which is uncommonly hot in Haldenvassdraget. Lamprey larval habitat can be divided in to three types. Type I is the preferred habitat, located in the dispositional zone of rivers with the sediment being a mixture of sand and fine organic matter. Type II which has a much lower density of larval consists generally of shifting sands and potentially contains gravel. Type III, which is considered an unacceptable habitat for lamprey, consists of hard packed gravel, hardpan clay and bedrock. Compared to other lamprey species, larvae of river and brook lamprey are more commonly found in medium/coarse sand. After the larva phase metamorphosis occurs and the life cycle diverges between the parasitic river and non-parasitic brook lamprey. Once they have completed their metamorphosis, river lamprey enters their parasitic juvenile phase, and travel downstream to wider regions of large rivers where they feed until they have grown large and migrate

upstream to spawn as adults. Brook lamprey does not have a juvenile parasitic phase. Their sexual maturation commences during the metamorphosis from larvae to adult. As an adult they lose the ability to feed, and spawning happens soon after sexual maturity (Docker, 2014). Spawning behaviour begins in spring to early summer. Upstream migrations occur around 7-8°C for both species, and they both spawn at 10-11°C (Schartum & Kraabøl, 2013). Both species die about two weeks after spawning (Docker, 2014). Landlocked in Haldenvassdraget and unable to migrate to the ocean, river lamprey joins the brook lamprey as a potamodromous fish (Docker, 2014; Hesthagen et al., 2021). Other freshwater populations of river lamprey are also known from other countries (Degerman, 2009; Goodwin et al., 2006).

## 1.3 Aim of study

The aim of this study is to assess detection probability of two different eDNA-based methods, the more established single-species qPCR and the newer, more complex multi-species DNA-metabarcoding, on elusive freshwater fish. River and brook lamprey in Haldenvassdraget were chosen as targeted species for the methods. In addition to assessing the methods, the results of this study will add to the knowledge of lamprey distribution within Haldenvassdraget.

# 2 Materials and methods

## 2.1 Study area

The study was conducted in the typical lowland river system, Haldenvassdraget (Figure 2). The system is a relatively large river-lake system in the south-east of Norway, with a length of 150 km, and a total catchment area of 1588 km2. Haldenvassdraget borders on three major river systems: in the north-east the Mangenvassdrag, in the west the large Glommavassdrag, and to the east Upperudsälven in Sweden (Johanson, 2002).


Figure 2. A: Map of northern Europe with Haldenvassdraget in blue. B: Map of Haldenvassdraget outlined in blue. Maps made in NVE Atlas

The main source of Haldenvassdraget springs from Dragsjøhaugen (268 m.a.s.l.) south of Årnes in Nes municipality. From Dragsjøhaugen the river system establishes the lakes Floangen and Floen, before it flows through Liermosen, one of Norway's largest peat bogs. It then continues through Bjørkelangen (124 m.a.s.l.), to Hølandselva ending in the lakes Skulerudsjøen and Rødenessjøen (118 m.a.s.l). The tributaries Hafsteinselva from Hemnessjøen (Øgderen) and Mjerma from Setten feed into Hølandselva. The geography of the river system changes after Skulerudsjøen, where it all the way to Halden can be categorized with short rivers connecting larger lakes. In quick succession there is Rødnessjøen (118 m.a.s.l.), Øymarksjøen (107 m.a.s.l), Aremarksjøen (105 m.a.s.l), Aspern (105 m.a.s.l) and Femsjøen (79 m.a.s.l). Haldenvassdraget ends in the river Tista which flows

out to Iddefjorden. The main river system is also joined by numerous smaller tributaries. (Johanson, 2002). Throughout the river system there are migration barriers for fish, notable ones include hydropower dams in Bjørkelangen, Ørje, Brekke, Strømsfoss and Tista (Johnsen & Vrålstad, 2009; Selbekk et al., 2020)

Due to human activity negatively affecting Haldenvassdraget continuously, measures to improve the water quality in Haldenvassdraget have been prioritised since the 1960s. The purpose of these measures put in place were to reduce nutrient supply to the river system from agriculture and treatment plants. In recent times monitoring has shown no clear trends in the development of water quality in the selected lakes. However, there is a clear connection between water quality and rainfall conditions, runoff, and the total phosphorus in the lakes (Greipsland et al., 2018).

The water quality is extremely varied in Haldenvassdraget. Water quality is quite poor in the upperparts, with the quality drastically improving downstream towards Iddefjorden. The upper parts of the main river and many of the tributaries are strongly affected by run-off from agriculture and households, causing a great deal of eutrophication, which gradually decreases through the system. The lakes filter out a lot of the dissolved excess nutrients. The characteristics of the lakes vary greatly, they come in all shapes and sizes, and nutrient richness (Spikkeland, 2014). Haldenvassdraget is located under the Marine limit, and heavily influenced by nutrient rich marine clay. Upstream from Ørje there is a steady supply of clay particles that are mainly responsible for the nutrient richness downstream. The amount of humus has increased in the whole river system the last 10 years. This is due to a decrease in acidification from acid rain (sulphur and nitrogen), and powerful precipitation resulting in leaching of humus into the river system. The variation in climate effects has a complex effect on the transport of total phosphorus and the algae development in the lakes, resulting in large variations in water quality from year to year (Greipsland et al., 2018).

Haldenvassdraget is considered species rich for Norway (Spikkeland, 2014). Fish migrating to Norway during the last ice age often ended up in Haldenvassdraget and did not migrate much further. The climate of the river system is also warmer than the rest of the country, which also contributes to a higher species richness (Huitfeldt-Kaas, 1918; Spikkeland, 2014). Fish species found in Haldenvassdraget include: sea lamprey, river lamprey, brook lamprey,

European eel (*Anguilla anguilla)*, northern pike (*Esox Lucius*), Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), Arctic char (*Salvelinus alpina*), vendace (*Coregonus Albula[FF1]* ), European whitefish (*Coregonus lavaretus*), European smelt (*Osmerus eperlanus*), roach (*Rutilus rutilus*), common dace (*Leuciscus leuciscus*), ide (*Leuciscus idus*), Eurasian minnow (*Phoxinus phoxinus*), rudd (*Scardinius erythrophthalmus*), asp (*Aspius aspius*), bleak (*Alburnus alburnus*), white bream (*Blicca bjoerkna*), freshwater bream (*Abramis brama*), crucian carp (*Carassius carassius*), common carp (*Cyprinus carpio*), burbot (*Lota lota*), three-spined stickleback (*Gasterosteus aculeatus*), bullhead (*Cottus gobio*), alpine bullhead (*Cottus poecilopus*), European pearch (*Perca fluviatilis*), pike-perch (*Stizostedion lucioperca*), and ruffe (*Gymnocephalus cernua*) (Spikkeland 2014).

## 2.2 eDNA sampling protocol

Haldenvassdragets' geography and local knowledge (from discussions with stakeholders) were considered when I was planning and conducting the study. Sampling locations were purposely selected to cover as much of the main river and tributaries as possible. Physical barriers, such as migration barriers hindering the movement of lampreys were also considered, in order to increase the possibility of detecting lamprey. Accessibility of the locations for sampling purposes were considered.

A total of 45 locations in Haldenvassdraget were visited and sampled from June 7$^{th}$ to June 13$^{th}$, 2021 (Figure 2, Appendix table 1). The locations consisted of nine samples from the main river, and 36 samples from different tributaries. Samples were taken when good weather conditions had persisted for multiple days.

eDNA sampling was performed using sterile equipment at each location to hinder contamination. Water was collected from the water edge, to hinder contamination from footwear. To increase the likelihood of finding eDNA from lamprey residing above the sampling location, samples were acquired from turbulent or fast-moving water. Surgical gloves were always worn and disposed of between locations.

Two water samples were collected and filtrated at each site. The volume of water that was filtrated at different locations varied from 1 to 5 L due to the particles filling up the filter at

different rates at each location. A 1000 mL water sampler (plastic bucket) was used to collect running water, a deposable silicone tube was used to pump the water through a NatureMetrics capsule filter, consisting of two filters, a 5.0 µm glass fibre filter, and a 0.8 µm PES main filter, by the help of a peristaltic pump (Bürkle Vampire). To avoid damage to the filter, the speed of the pump was set to 1. At the end of each filtration, the capsule was emptied of water and dried.

A syringe with preservative buffer (ATL-buffer, Qiagen) was screwed onto the inlet of the capsule, holding the syringe and capsule upright so the outlet pointed up, the buffer was slowly introduced to the filter, until the filter was completely saturated with buffer, indicated with a droplet emerging on the outlet of the filter. About 1.5 mL ATL-buffer (Qiagen) was added to each capsule. A luer cap was then screwed on the inlet while the filter was pointing up. After that, with the filter pointing down, the syringe was unscrewed and a luer cap was screwed on the outlet. The filters were then labelled with number, date, location and station in chronological order.

Temperature was measured using a digital thermometer at each location after the samples were collected.

The filters were stored at dark conditions and room temperature (above 10°C), to prevent crystallization, due to the preservation buffer in the syringe being temperature sensitive. The samples were then transported to the Centre for Biodiversity Genetics (NINAGEN) at NINA in Trondheim for genetic analyses.

## 2.3 DNA – extraction

DNA-extraction, qPCR and library prep for DNA-metabarcoding were done by professional lab technicians at the Norwegian Centre for Biodiversity Genetics (NINAGEN) in Trondheim. For the DNA-extraction, 450 µL Proteinase-K (Qiagen) was added to the sampling tubes and incubated overnight at 56°C. DNA was isolated using NucleoSpin Plant II Midi kit (Macherey-Nagel), following the manufacturers protocol except that Qiagen buffers were used instead of those supplied with the kit. Extracted DNA was eluted in 200 µL AE buffer. Samples were re-eluted for maximizing the output of DNA.

## 2.4 qPCR analysis

The species-specific genetic markers cannot differentiate between river and brook lamprey as of now, they are therefore considered ecotypes when analysed using quantitative PCR (qPCR) for detection of the target species using the LampATPase6_1 assay (Zancolli et al., 2018). Each qPCR-reaction had a total volume of 30 µL which included 15 µL TaqMan Fast Advanced Master Mix (ThermoFisher Scientific), 0.9 µM of forward, reverse primer and probe, 4.5 dH20 and 5 µL DNA-template. PCR-conditions started with an onset of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 90 sec, and were finalized with 72°C for 10 min. All samples were analysed using a QuantStudio 5 qPCR-machine (ThermoFisher Scientific), and all samples were analysed in triplicates where only samples showing at least 2 out of 3 positive results were considered positive for the target species.

## 2.5 Metabarcoding analysis

For DNA-metabarcoding of fish, the 12S region was amplified using the MiFish-U-F and MiFish-U-R primers (Miya et al., 2015). PCR was conducted in 25 µL volumes containing: 1X KAPA HiFi HotStart Ready Mix, 0.3 µM of each primer, and 2.5 µL of 10 ng/µL template DNA. The PCR conditions were as follows; first a denaturation step of 95°C for 3 minutes, followed by 35 cycles of: 98°C for 20 seconds, annealing at 65°C for 15 seconds, and elongation at 72°C for 15 seconds. The PCR were finalized with 72°C for 5 minutes.

Amplicons were quality checked on a Tape Station (Agilent 4200) and cleaned with magnetic beads (MAG-BIND RXN PURE PLUS) to remove fragments <200bp after each PCR. Indexes for Illumina DNA/RNA UD indices were added to the 5' and 3' ends of the amplicons according to the manufacturer's instructions. Amplicons were diluted to 6 ng/µL, and magnetic beads were used to remove fragments <500bp. Amplicons were pooled in equimolar amounts and sequenced on an Illumina NovaSeq 6000 platfrom at the Norwegian Sequencing Centre (NSC) in Oslo.

## 2.6 DNA – metabarcoding bioinformatics

Demultiplexing was performed on the Illumina NovaSeq platform. Primer removal was conducted with cutadapt v. 1.9.1 requiring a minimum length match of 17 bp with 0.15 expected errors. Quality filtering, error correction, merging, mapping and chimera removal were conducted in DADA2 (Callahan et al., 2016) to generate ASV (Amplicon Sequence Variants). ASV, the quality assured DNA-sequences, were used to separate the species, as well as investigation genetic variation within species. Taxonomy was assigned using BLASTN comparisons to the GenBank database. A minimum of 97% identity and 90% coverage with a reference sequence were required for a successful assignment at the species level.

## 2.7 Statistical analyses

I used generalised linear mixed models (GLMM) for testing whether the two methods, qPCR and DNA-metabarcoding, differed in detection probability of lamprey. I included water volume and temperature in addition to method as fixed factors. Sampling location was added as a random factor to control for non-independence of samples collected at the same site. Water volume was considered due to larger water volumes previously being found to increase detection probability (Agersnap et al., 2017; Muha et al., 2019). Water temperature has been shown to affect the amount of eDNA released by fish, where more eDNA was released at higher temperatures (Lacoursière-Roussel et al., 2016; Wacker et al., 2019). I used the package lmerTest (Kuznetsova et al., 2017) in the statistical software R (Team, 2021) to run the mixed models.

# 3 Results

## 3.1 qPCR

All samples were analysed in triplicates with a threshold set of at least 2 out of 3 positive results to be considered positive for lamprey. As shown in Table 1, 37 of 90 samples showed presence of lamprey, resulting in lamprey being detected at 20 of 45 location. 52 of 90 samples showed no presence of lamprey, resulting in lamprey not being detected at 25 of 45 location. Sample 14B Upper Mjerma failed, due to the lid of the tube that the sample was in, opened while in the centrifuge, and was lost.

At some locations there were differences between the two replicate samples. Hemneselva (07) showed some differences in presence, with sample A showing presence in 3/3 replicates and sample B showing presence in 2/3 replicates. There was also a difference within Lower Mjerma (15), where sample A indicated no presence (1/3 positive replicates) and sample B showing presence (3/3 positive replicates). Other locations where there were differences between samples was Dalselva and Lower Hafsteinselva, where sample A showed no presence (0/3 positive replicates) and samples B showed presence (3/3 positive replicates). If either one of the two samples are positive, I accept the location as positive for presence of lamprey.

For qPCR all positive controls were positive and negative controls were negative.

Table 1 Results from qPCR analysis showing sample ID, locality, number of replicates and how many of the replicas tested positive for lamprey DNA. $C_T$ is PCR cycles. Lower $C_T$ Mean equates to higher concentration of DNA. $C_T$ SD is the standard deviation of $C_T$.

| Sample ID | Locality | No. replicates | No. positive replicates | Ct Mean | Ct SD |
|---|---|---|---|---|---|
| 01A | Ulviksjøen | 3 | 1 | 37.10 | |
| 01B | Ulviksjøen | 3 | 0 | | |
| 02A | Eidsbekken | 3 | 3 | 27.99 | 0.13 |
| 02B | Eidsbekken | 3 | 3 | 27.96 | 0.05 |
| 03A | Lierelva | 3 | 3 | 39.34 | 1.42 |
| 03B | Lierelva | 3 | 3 | 33.93 | 0.89 |
| 04A | Snartjern | 3 | 3 | 27.93 | 0.13 |
| 04B | Snartjern | 3 | 3 | 28.18 | 0.08 |

| | | | | | |
|---|---|---|---|---|---|
| 05A | Malttjernbekken | 3 | 3 | 28.97 | 0.03 |
| 05B | Malttjernbekken | 3 | 3 | 29.41 | 0.13 |
| 06A | Dalselva | 3 | 0 | | |
| 06B | Dalselva | 3 | 3 | 27.80 | 0.24 |
| 07A | Hemneselva | 3 | 3 | 34.46 | 0.93 |
| 07B | Hemneselva | 3 | 2 | 37.59 | 0.77 |
| 08A | Lower Hafsteinselva | 3 | 0 | | |
| 08B | Lower Hafsteinselva | 3 | 3 | 28.25 | 0.14 |
| 09A | Hølandselva | 3 | 3 | 29.67 | 0.19 |
| 09B | Hølandselva | 3 | 3 | 31.18 | 0.15 |
| 10A | Upper Hafsteinselva | 3 | 3 | 24.64 | 0.06 |
| 10B | Upper Hafsteinselva | 3 | 3 | 31.08 | 8.64 |
| 11A | Setta | 3 | 0 | | |
| 11B | Setta | 3 | 0 | | |
| 12A | Langebruslora | 3 | 0 | | |
| 12B | Langebruslora | 3 | 0 | | |
| 13A | Langtjen | 3 | 0 | | |
| 13B | Langtjen | 3 | 1 | 37.69 | |
| 14A | Upper Mjerma | 3 | 0 | | |
| 14B | Upper Mjerma | - | - | | |
| 15A | Lower Mjerma | 3 | 1 | 34.89 | |
| 15B | Lower Mjerma | 3 | 3 | 28.42 | 0.25 |
| 16A | Nautebrofoss | 3 | 3 | 28.49 | 0.32 |
| 16B | Nautebrofoss | 3 | 3 | 26.87 | 0.26 |
| 17A | Østenbyelva | 3 | 0 | | |
| 17B | Østenbyelva | 3 | 0 | | |
| 18A | Taraldruelva | 3 | 3 | 30.97 | 4.36 |
| 18B | Taraldruelva | 3 | 3 | 25.40 | 0.14 |
| 19A | Åsebyelva | 3 | 3 | 35.62 | 0.98 |
| 19B | Åsebyelva | 3 | 3 | 35.22 | 0.67 |
| 20A | Langnes | 3 | 3 | 28.62 | 0.05 |
| 20B | Langnes | 3 | 3 | 29.04 | 0.18 |
| 21A | Engerelva | 3 | 3 | 7.626 | 0.07 |
| 21B | Engerelva | 3 | 3 | 28.08 | 0.10 |
| 22A | Ørje | 3 | 0 | | |
| 22B | Ørje | 3 | 1 | 44.14 | |
| 23A | Bøenselva | 3 | 0 | | |
| 23B | Bøenselva | 3 | 0 | | |
| 24A | Gunnengbekken | 3 | 3 | 37.60 | 1.30 |
| 24B | Gunnengbekken | 3 | 3 | 36.33 | 1.45 |
| 25A | Skinnarbutjenn | 3 | 0 | | |
| 25B | Skinnarbutjenn | 3 | 0 | | |
| 26A | Langetjernelva | 3 | 0 | | |
| 26B | Langetjernelva | 3 | 0 | | |
| 27A | Fangebekken | 3 | 3 | 32.34 | 0.25 |
| 27B | Fangebekken | 3 | 3 | 31.71 | 0.08 |

| | | | | | |
|---|---|---|---|---|---|
| 28A | Fossby | 3 | 1 | 28.65 | |
| 28B | Fossby | 3 | 0 | | |
| 29A | Skolleborg | 3 | 0 | | |
| 29B | Skolleborg | 3 | 0 | | |
| 30A | Verksbrua | 3 | 0 | | |
| 30B | Verksbrua | 3 | 0 | | |
| 31A | Tenebekken | 3 | 0 | | |
| 31B | Tenebekken | 3 | 0 | | |
| 32A | Strømsfoss | 3 | 0 | | |
| 32B | Strømsfoss | 3 | 0 | | |
| 33A | Lielva | 3 | 3 | 34.92 | 1.19 |
| 33B | Lielva | 3 | 3 | 34.11 | 0.22 |
| 34A | Valbyelva | 3 | 0 | | |
| 34B | Valbyelva | 3 | 0 | | |
| 35A | Holmegilelva | 3 | 0 | | |
| 35B | Holmegilelva | 3 | 0 | | |
| 36A | Kverntjern | 3 | 0 | | |
| 36B | Kverntjern | 3 | 0 | | |
| 37A | Stenselva | 3 | 0 | | |
| 37B | Stenselva | 3 | 0 | | |
| 38A | Ganerødelva | 3 | 0 | | |
| 38B | Ganerødelva | 3 | 0 | | |
| 39A | Mellebyelva | 3 | 0 | | |
| 39B | Mellebyelva | 3 | 0 | | |
| 40A | Rødselva | 3 | 0 | | |
| 40B | Rødselva | 3 | 1 | 41.71 | |
| 41A | Rjørelva | 3 | 0 | | |
| 41B | Rjørelva | 3 | 0 | | |
| 42A | Asakbekken | 3 | 3 | 27.94 | 0.09 |
| 42B | Asakbekken | 3 | 3 | 27.98 | 0.12 |
| 43A | Tistedalsfoss | 3 | 3 | 37.23 | 1.57 |
| 43B | Tistedalsfoss | 3 | 3 | 40.99 | 2.63 |
| 44A | Skåningsfoss | 3 | 0 | | |
| 44B | Skåningsfoss | 3 | 0 | | |
| 45A | Porsnes sluser | 3 | 0 | | |
| 45B | Porsnes sluser | 3 | 0 | | |

## 3.2 DNA – metabarcoding

There was large variation in the number of total reads for DNA-metabarcoding between samples (Appendix table 1). Percentage of detected lamprey relative reads varied between less than 1% to 47% (Table 2). Most samples were acceptable for the number of reads, but for the following location the total reads were quite low seen in context to lamprey relative reads, like in Asakbekken (42B) 0.47/5480.

As shown in Table 2, 31 of 90 samples were positive for lamprey, indicating that lamprey was present in 19 of 45 locations. Lamprey was not present in 43 of 90 samples, indicating that lamprey was not present in 24 of 45 locations. 16 of 90 samples failed, but there were only two locations where both sample A and B failed. This equates to 17.$\dot{7}$ % of the samples not working correctly. These samples showed presences for bacteria and fungi but lacked freshwater fish DNA.

Within 18 locations there were differences between the two replicate samples. Both replicate samples failed in Upper Mjerma (14) and Holmegilelva (35). For Rødselva (40), one sample failed and the other showed presence of lamprey. In 11 locations one sample failed, and the other showed no presence of lamprey. The locations were Setta (11), Østenbyelva (17), Langtjernelva (26), Fossby (28), Skolleborg (29), Verksbrua (30), Tenebekken (31), Kverntjern (36), Stenselva (37), Mellebyelva (39) and Skåningsfoss (44).

Both positive and negative controls for river/brook lamprey worked.

Table 2 Number of reads per sample after DNA-metabarcoding and bioinformatic analyses (see more in Appendix table 1). No. fish reads, is the total number of fish reads in the sample. No. lamprey reads, is how many reads of the No. fish reads were lamprey reads. Lamprey relative reads is relative lamprey reads. And result show how many samples tested positive for lamprey.

| Sample ID | Locality | No. fish reads | No. lamprey reads | Lamprey relative reads | Result |
|---|---|---|---|---|---|
| 01A | Ulviksjøen | 123143 | 0 | 0 | NEG |
| 01B | Ulviksjøen | 108306 | 0 | 0 | NEG |
| 02A | Eidsbekken | 524914 | 3362 | 0.01 | POS |
| 02B | Eidsbekken | 554446 | 4463 | 0.01 | POS |
| 03A | Lierelva | 128354 | 4991 | 0.04 | POS |
| 03B | Lierelva | 136224 | 1522 | 0.01 | POS |
| 04A | Snartjern | 296624 | 2855 | 0.01 | POS |

| | | | | | |
|---|---|---|---|---|---|
| 04B | Snartjern | 339131 | 1969 | 0.01 | POS |
| 05A | Maltjennbekken | 137131 | 2717 | 0.02 | POS |
| 05B | Maltjennbekken | 173915 | 1614 | 0.01 | POS |
| 06A | Dalselva | 117960 | 11297 | 0.10 | POS |
| 06B | Dalselva | 58284 | 2891 | 0.05 | POS |
| 07A | Hemneselva | 141659 | 95 | 0.00 | POS |
| 07B | Hemneselva | 36005 | 0 | 0 | NEG |
| 08A | Lower Hafsteinselva | 18588 | 0 | 0 | NEG |
| 08B | Lower Hafsteinselva | 20196 | 654 | 0.03 | POS |
| 09A | Hølandselva | 61200 | 1094 | 0.02 | POS |
| 09B | Hølandselva | 32323 | 54 | 0.00 | POS |
| 10A | Upper Hafsteinselva | 252563 | 33295 | 0.13 | POS |
| 10B | Upper Hafsteinselva | 149066 | 21099 | 0.14 | POS |
| 11A | Setta | 84863 | 0 | 0 | NEG |
| 11B | Setta | 0 | NA | NA | NA |
| 12A | Langebruslora | 30620 | 0 | 0 | NEG |
| 12B | Langebruslora | 26481 | 0 | 0 | NEG |
| 13A | Langtjen | 18980 | 0 | 0 | NEG |
| 13B | Langtjen | 44228 | 0 | 0 | NEG |
| 14A | Upper Mjerma | 0 | NA | NA | NA |
| 14B | Upper Mjerma | 0 | NA | NA | NA |
| 15A | Lower Mjerma | 143222 | 672 | 0.00 | POS |
| 15B | Lowe Mjerma | 96479 | 2145 | 0.02 | POS |
| 16A | Nautebrofoss | 59424 | 4514 | 0.08 | POS |
| 16B | Nautebrofoss | 93011 | 5351 | 0.06 | POS |
| 17A | Østenbyelva | 0 | NA | NA | NA |
| 17B | Østenbyelva | 54421 | 0 | 0 | NEG |
| 18A | Taraldruelva | 81966 | 16275 | 0.20 | POS |
| 18B | Taraldruelva | 78025 | 21135 | 0.27 | POS |
| 19A | Åsebyelva | 14715 | 139 | 0.01 | POS |
| 19B | Åsebyelva | 7642 | 0 | 0 | NEG |
| 20A | Langnes | 126497 | 429 | 0.00 | POS |
| 20B | Langnes | 168234 | 0 | 0 | NEG |
| 21A | Engerelva | 163123 | 4613 | 0.03 | POS |
| 21B | Engerelva | 195665 | 3117 | 0.02 | POS |
| 22A | Ørje | 38584 | 0 | 0 | NEG |
| 22B | Ørje | 33260 | 0 | 0 | NEG |
| 23A | Bøenselva | 46218 | 0 | 0 | NEG |
| 23B | Bøenselva | 36906 | 0 | 0 | NEG |
| 24A | Gunnengbekken | 11597 | 24 | 0.00 | POS |
| 24B | Gunnengbekken | 20850 | 0 | 0 | NEG |
| 25A | Skinnarbutjenn | 52248 | 0 | 0 | NEG |
| 25B | Skinnarbutjenn | 82281 | 0 | 0 | NEG |
| 26A | Langetjernelva | 0 | NA | NA | NA |
| 26B | Langetjernelva | 91083 | 0 | 0 | NEG |
| 27A | Fangebekken | 195522 | 0 | 0 | NEG |

| | | | | | |
|---|---|---|---|---|---|
| 27B | Fangebekken | 145248 | 18 | 0.00 | POS |
| 28A | Fossby | 206737 | 0 | 0 | NEG |
| 28B | Fossby | 0 | NA | NA | NA |
| 29A | Skolleborg | 543 | 0 | 0 | NEG |
| 29B | Skolleborg | 8255 | 0 | 0 | NEG |
| 30A | Verksbrua | 0 | NA | NA | NA |
| 30B | Verksbrua | 61726 | 0 | 0 | NEG |
| 31A | Tenebekken | 0 | NA | NA | NA |
| 31B | Tenebekken | 3537 | 0 | 0 | NEG |
| 32A | Strømsfoss | 75699 | 0 | 0 | NEG |
| 32B | Strømsfoss | 40798 | 0 | 0 | NEG |
| 33A | Lielva | 160183 | 0 | 0 | NEG |
| 33B | Lielva | 221201 | 0 | 0 | NEG |
| 34A | Valbyelva | 155144 | 0 | 0 | NEG |
| 34B | Valbyelva | 68240 | 0 | 0 | NEG |
| 35A | Holmegilelva | 0 | NA | NA | NA |
| 35B | Holmegilelva | 0 | NA | NA | NA |
| 36A | Kverntjern | 40398 | 0 | 0 | NEG |
| 36B | Kverntjern | 0 | NA | NA | NA |
| 37A | Stenselva | 20758 | 0 | 0 | NEG |
| 37B | Stenselva | 606 | 0 | 0 | NEG |
| 38A | Ganerødelva | 12855 | 0 | 0 | NEG |
| 38B | Ganerødelva | 24760 | 0 | 0 | NEG |
| 39A | Mellebyelva | 41782 | 0 | 0 | NEG |
| 39B | Mellebyelva | 0 | NA | NA | NA |
| 40A | Rødselva | 0 | NA | NA | NA |
| 40B | Rødselva | 48345 | 415 | 0.01 | POS |
| 41A | Rjørelva | 39359 | 0 | 0 | NEG |
| 41B | Rjørelva | 14532 | 0 | 0 | NEG |
| 42A | Asakbekken | 97710 | 3879 | 0.04 | POS |
| 42B | Asakbekken | 5480 | 2594 | 0.47 | POS |
| 43A | Tistedalsfoss | 19947 | 0 | 0 | NEG |
| 43B | Tistedalsfoss | 19686 | 0 | 0 | NEG |
| 44A | Skåningsfoss | 0 | NA | NA | NA |
| 44B | Skåningsfoss | 7980 | 0 | 0 | NEG |
| 45A | Porsnes sluser | 31134 | 0 | 0 | NEG |
| 45B | Porsnes sluser | 19765 | 0 | 0 | NEG |

## 3.3 Comparing qPCR and metabarcoding for detecting lampreys

The combined the results of qPCR (Table 1) and DNA-metabarcoding (*Table 2*) results in 21 locations of lamprey presence and 24 locations of lamprey absence (Table 3). If at least one sample per location tested positive for lamprey in either of these methods, lamprey was considered present in that location.

DNA-metabarcoding showed no fish in 16 samples, but OD measurements, a measurement used to detect contaminants, show decent quality DNA in the DNA-extracts (Appendix table 2). It is unlikely that there were no fish in these locations, and the results from the qPCR analyses also came back negative for all 16 samples. It is therefore not possible to differentiate whether these samples also did not work for the qPCR analyses, or if they were just negative for the presence of lamprey.

Table 3 Summery of Table 1 and 2 of lamprey in Haldenvassdraget based on eDNA-samples analysed using qPCR (sample A and B) or DNA-metabarcoding – Bar (sample A and B). If lamprey was detected using one of the methods, presences was accepted as true for the location.

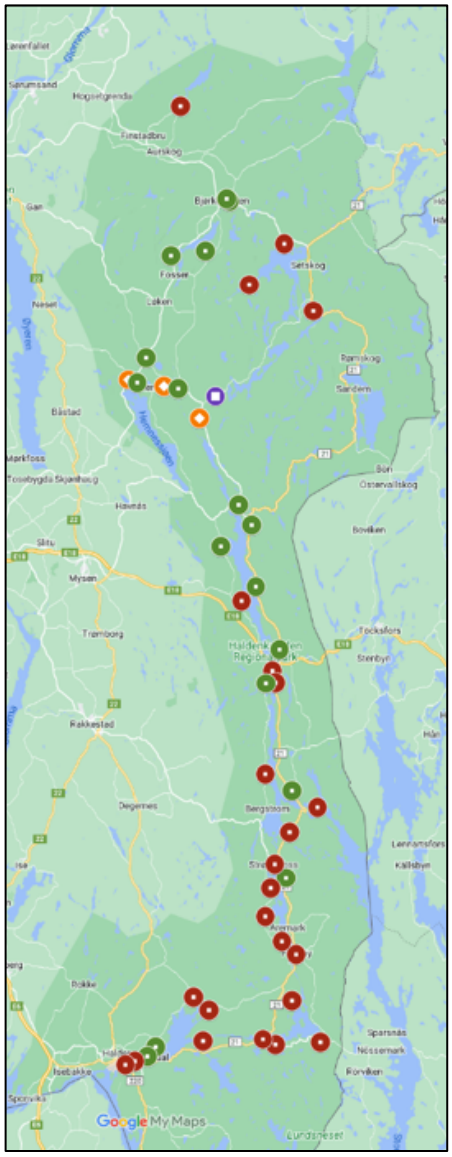
| # | Locality | qPCR A | qPCR B | Bar A | Bar B | Presence |
|---|---|---|---|---|---|---|
| 1 | Ulviksjøen | NEG | NEG | NEG | NEG | ABSENT |
| 2 | Eidsbekken | POS | POS | POS | POS | PRESENT |
| 3 | Lierelva | POS | POS | POS | POS | PRESENT |
| 4 | Snartjern | POS | POS | POS | POS | PRESENT |
| 5 | Maltjennbekken | POS | POS | POS | POS | PRESENT |
| 6 | Dalselva | NEG | POS | POS | POS | PRESENT |
| 7 | Hemneselva | POS | POS | POS | NEG | PRESENT |
| 8 | Lower Hafsteinselva | NEG | POS | NEG | POS | PRESENT |
| 9 | Hølandselva | POS | POS | POS | POS | PRESENT |
| 10 | Upper Hafsteinselva | POS | POS | POS | POS | PRESENT |
| 11 | Setta | NEG | NEG | NEG | - | ABSENT |
| 12 | Langebruslora | NEG | NEG | NEG | NEG | ABSENT |
| 13 | Langtjenn | NEG | NEG | NEG | NEG | ABSENT |
| 14 | Upper Mjerma | NEG | - | - | - | ABSENT |
| 15 | Lower Mjerma | NEG | POS | POS | POS | PRESENT |
| 16 | Nautebrofoss | POS | POS | POS | POS | PRESENT |
| 17 | Østenbyelva | NEG | NEG | - | NEG | ABSENT |

| | | | | | | |
|---|---|---|---|---|---|---|
| 18 | Taraldrudelva | POS | POS | POS | POS | PRESENT |
| 19 | Åsebyelva | POS | POS | POS | NEG | PRESENT |
| 20 | Langnes | POS | POS | POS | NEG | PRESENT |
| 21 | Engerelva | POS | POS | POS | POS | PRESENT |
| 22 | Ørje | NEG | NEG | NEG | NEG | ABSENT |
| 23 | Bøenselva | NEG | NEG | NEG | NEG | ABSENT |
| 24 | Gunnengbekken | POS | POS | POS | NEG | PRESENT |
| 25 | Skinnarbutjern | NEG | NEG | NEG | NEG | ABSENT |
| 26 | Langetjernelva | NEG | NEG | - | NEG | ABSENT |
| 27 | Fangebekken | POS | POS | NEG | POS | PRESENT |
| 28 | Fossby | NEG | NEG | NEG | - | ABSENT |
| 29 | Skolleborg | NEG | NEG | - | NEG | ABSENT |
| 30 | Verksbrua | NEG | NEG | - | NEG | ABSENT |
| 31 | Tenebekken | NEG | NEG | - | NEG | ABSENT |
| 32 | Strømsfoss | NEG | NEG | NEG | NEG | ABSENT |
| 33 | Lielva | POS | POS | NEG | NEG | PRESENT |
| 34 | Valbyelva | NEG | NEG | NEG | NEG | ABSENT |
| 35 | Holmegilelva | NEG | NEG | - | - | ABSENT |
| 36 | Kverntjern | NEG | NEG | NEG | - | ABSENT |
| 37 | Stenselva | NEG | NEG | NEG | - | ABSENT |
| 38 | Ganerødelva | NEG | NEG | NEG | NEG | ABSENT |
| 39 | Mellebyelva | NEG | NEG | NEG | - | ABSENT |
| 40 | Rødselva | NEG | NEG | - | POS | PRESENT |
| 41 | Rjørelva | NEG | NEG | NEG | NEG | ABSENT |
| 42 | Asakbekken | POS | POS | POS | POS | PRESENT |
| 43 | Tistedalsfoss | POS | POS | NEG | NEG | PRESENT |
| 44 | Skåningsfoss | NEG | NEG | - | NEG | ABSENT |
| 45 | Porsnes sluser | NEG | NEG | NEG | NEG | ABSENT |

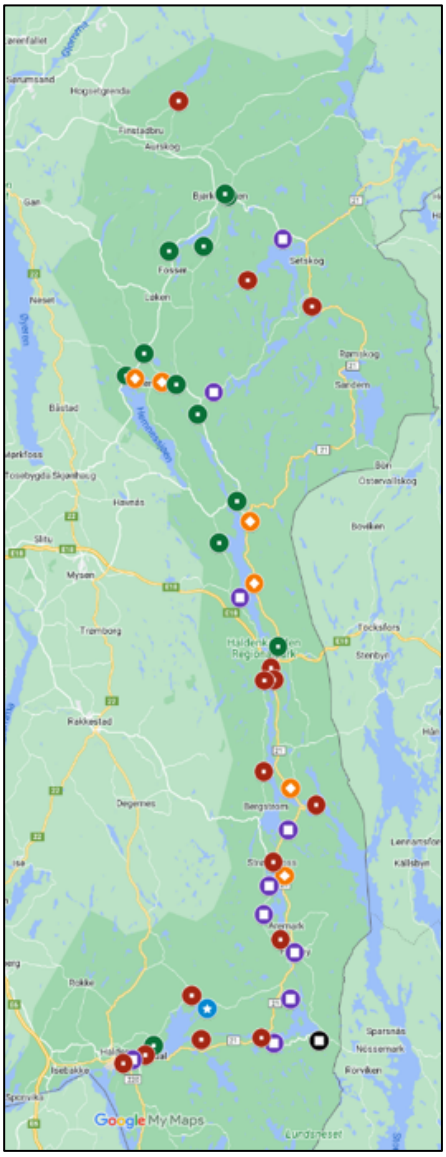
Considering Table 3 and Figure 3 it is clear that there is no presence of lamprey eDNA at the sampling starting point Ulviksjøen, but it was detected in the main river leading to Bjørkelangen, and the tributaries connecting to the lake. The main rivers connecting to Hemnessjøen and Rødenessjøen through Hølandselva also showed presence. One tributary, west connecting to Rødenessjøen showed no presence. No lamprey was detected in any of the location in the tributary Setten but lamprey was detected below the waterfall from Setten connecting to the main river Hølandselva. No lamprey was detected in Ørje river, connecting Rødenessjøen and Øymarksjøen. Two tributaries connecting to Øymarksjøen show lamprey presence, one on the northwest side of the lake and the other on the east side. Only one tributary connecting to Aremarksjøen, in the northeast, showed lamprey presence, no presence

was detected in the main river. No presence was detected throughout Aspen, Stenselva and tributary Ganerødelva, connecting to Store Erte. In Femsjøen, only the tributary Asakbekken showed lamprey presence. The last location where lamprey was present was Tistedalsfoss. No presence was found downstream after Porsnes sluser, which marks the end of my sampling of Haldenvassdraget.

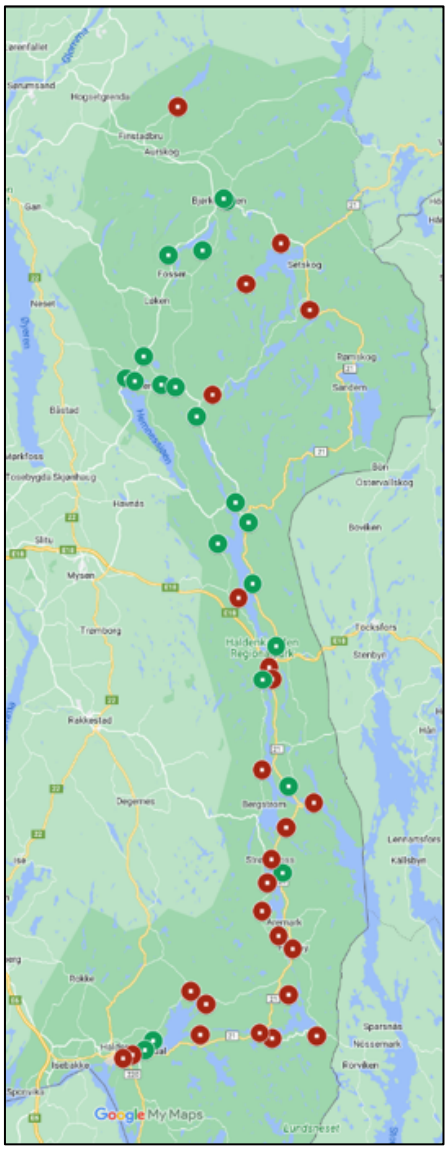
Based on the map (Figure 3 C) it would seem that the upper parts of Haldenvassdraget contain more lamprey than in the lower part of the system, and that there is no lamprey in the northeast tributary Setten.

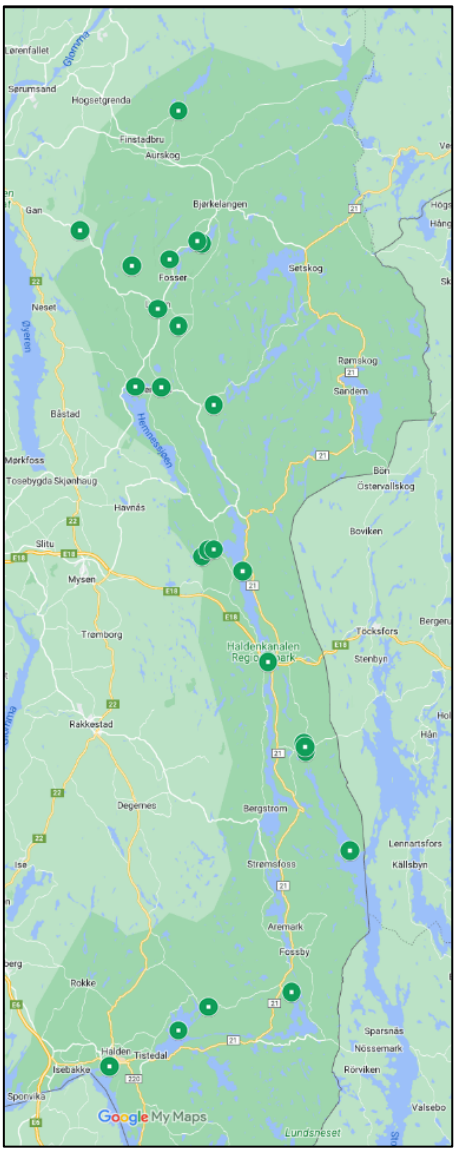



A: qPCR result




B: DNA-metabarcoding result




C: Results combined

D: Prior observations

Figure 3 The maps show the distribution of lamprey presence in Haldenvassdraget for A: qPCR, B: DNA-metabarcoding and C: qPCR and DNA-metabarcoding combined. Green: lamprey DNA present in both samples, Red: lamprey DNA absent from both samples, Orange: lamprey DNA present in one sample and absent in the other, Purple: absence of lamprey DNA in one sample and one failed sample, Blue: presence of lamprey DNA in one sample and one failed sample, Black: both samples failed. In C: Green: lamprey DNA present, and Red: lamprey DNA absent. D: Prior observation from (Hesthagen et al., 2021; Spikkeland & Kasbo, 2014) and Artsdatabanken (Artskart Artsdatabanken). Maps made in Google.

## 3.4 Probability of detecting lamprey

To test if there was a difference in detection probability of lamprey between the two methods, qPCR and DNA-metabarcoding, generalized linear mixed models (GLMM) was used. Analysis was run on two different sets of data, henceforth data set A and data set B. In A, the 16 DNA-metabarcoding samples which had failed were removed and in B, in addition to the removal of failed DNA-metabarcoding samples, the corresponding qPCR results, which were all negative, were removed.

The differences in detection probability of lamprey between the different methods had no significant differences in A (Table 4A), however in B qPCR was statistically significantly better than DNA-metabarcoding (Table 4B). Both models show that water volume had a negative effect on detection probability, and water temperature had no significant effect.

Table 4 Model output from GLMM on the probability of detecting lamprey. The eDNA detection methods (qPCR and DNA-metabarcoding) are the main factors, with water temperature and volume as co-variables. A is testing with omitted failed DNA-metabarcoding results, and B is testing with omitted corresponding qPCR results.

| **A** | **Estimate** | **SE** | **Z** | **P** |
|---|---|---|---|---|
| (Intercept) | 8.72 | 7.71 | 1.13 | 0.26 |
| DNA-method (qPCR) | 0.88 | 0.63 | 1.39 | 0.17 |
| Water temperature | -0.42 | 0.44 | -0.96 | 0.34 |
| Water volume | -1.9 | 0.79 | -2.42 | 0.016 |
| **B** | **Estimate** | **SE** | **Z** | **P** |
| (Intercept) | 2.71 | 3.24 | 0.84 | 0.40 |
| DNA-method (qPCR) | 1.44 | 0.50 | 2.86 | 0.004 |
| Water temperature | -0.10 | 0.18 | -0.54 | 0.59 |
| Water volume | -0.84 | 0.39 | -2.15 | 0.032 |

# 4 Discussion

## 4.1 Detection performance of qPCR and DNA-metabarcoding

In this study I compared the detection sensitivity of qPCR and DNA-metabarcoding for lamprey in 45 locations, with two samples collected from each location. I compared if the samples were positive or negative for lamprey DNA within each method, and then compared the result from both methods to investigate presence and absence of lamprey for each location. The results suggest that both methods can detect lamprey, and the results from both methods show a similar spatial distribution for lampreys (Table 3, Figure 3).

To compare the methods, I checked for statistically significant differences by running a GLMM on the probability of detecting lamprey, as well as covariables, water temperature and water volume (Table 4). In DNA-metabarcoding, 16 of 90 samples failed, having no fish matches, suggesting no freshwater fish DNA in the samples. There was however DNA-sequences from non-target species, mainly represented by fungi DNA and bacteria DNA. There is no way of knowing what caused the samples to fail during analysis. It could be anything from amplification bias (Bylemans et al., 2019; Elbrecht & Leese, 2015; Kelly et al., 2019; Tremblay et al., 2015), amplification of non-target-species DNA (Alberdi et al., 2018; Gargan et al., 2022; Nichols et al., 2018; Piñol et al., 2019), mismatches with primer/template (Miya et al., 2020) or even PCR inhibition (Jane et al., 2015). These 16 DNA-metabarcoding results were discarded and the remaining 74 along with the 90 qPCR results comprised dataset A. The differences in detection probability of lamprey between the different methods had no significant differences in dataset A (Table 4A).

Interestingly, for all 16 samples of DNA-metabarcoding that failed to find fish DNA, the qPCR of those samples were negative for lamprey. This means that the qPCR for these samples might also have failed. There is no way of telling whether these qPCR samples are in fact negative, or if there were problems with the analyses. This uncertainty changes the main conclusion of my work. Removing these 16 samples also from qPCR results, the remaining 74 results along with the 74 DNA-metabarcoding results comprised dataset B. In this dataset there was a significant difference in detection probability of lamprey between the different methods, favouring qPCR over DNA-metabarcoding (Table 4B). This finding aligns with the

consensus that qPCR is a more sensitive method (Bylemans et al., 2019; Lecaudey et al., 2019; Schenekar et al., 2020). For a more reliable comparison, a positive control for qPCR analysis for a targeted species on each sample would be beneficial.

Both models showed that water volume had a negative effect on detection probability, and water temperature had no significant effect. This result is initially counter intuitive, due to higher water volume leading to more total eDNA in samples for a given concentration of eDNA (Bessey et al., 2020). There is no way of knowing why water volume had a negative effect on detection probability of lamprey in Haldenvassdraget. The ability of eDNA to attach to or be contained within particles is known to affect the chance to detect targeted species (Turner et al., 2014). An unknown factor is how well lamprey DNA attaches to organic materials, materials which might clog filters faster, hence leading to less water being filtered. From my combined results of locations of lamprey presence, the lampreys in Haldenvassdraget resided in areas with higher eutrophication and humus, so the preferred habitat by lampreys in Haldenvassdraget might contain more organic matter that lamprey DNA can attach to and clog filters faster.

A more general reason for why large water volume is not strictly needed, is that eDNA analyses methods are quite sensitive, so reaching the minimum required amount of DNA for eDNA analyses methods to conclude a positive result, is well within reason for 1L sample volume as it is for 5L sample volume. Previous studies have cited both 1L and 5L as sufficient volumes when sampling for eDNA (Harper et al., 2018; Yu et al., 2022). The volumes of my samples, 1L to 5L, might also not be a large enough sampling range to conclude anything decisively and the sample volumes are not distributed equally over the range, as there are much more sample volumes of 1L, or close to, than there are of sample volumes of 5L. My dataset of sample volumes being heavily skewed to the lower end, not only makes results more biased, but also means that upscaling or down scaling sample volumes, or changing sample location, will not necessarily yield similar connection of lower volume increasing detection chance.

Excluding the failed samples and looking at the remaining 74 samples, 37 out of 74 qPCR results were positive for lamprey, resulting in lamprey being detected at 20 locations (Table 1). Furthermore, 31 out of 74 DNA-metabarcoding results were positive for lamprey, resulting in lamprey being detected at 19 of 45 locations (Table 2).

Three locations stood out where qPCR and DNA-metabarcoding showed contradictive results. These locations were Lielva (33), Rødselva (40) and Tistedalsfoss (43). For Lielva (33), and Tistedalsfoss (43) qPCR detected lamprey while DNA-metabarcoding did not. For Rødselva (40) qPCR detected no lamprey DNA, however DNA-metabarcoding did detect lamprey. This is unexpected as qPCR is regarded as being more sensitive. Previous studies support qPCR having a higher detection sensitivity than DNA-metabarcoding when using fish as targeted species (Bylemans et al., 2019; Lecaudey et al., 2019; Schenekar et al., 2020). This is also true for other comparison studies where aquatic animals, such as great crested newt (*Triturus cristatus*) and Mediterranean fanworm (*Sabella spallanzanii*), were used (Harper et al., 2018; Wood et al., 2019).

With some samples failing it is important to assess the quality of this study. The sampling protocol I used while sampling was developed by NINA with the intent to standardise sampling, but also to make it easy enough to be used by citizen scientists without formal training. There was however there is no negative control.

The possibility of false positive and false negative results was considered, and steps were taken to minimise the risk of them occurring. For both qPCR and DNA-metabarcoding, water was used as a negative control and lamprey DNA was used as a positive control. The positive and negative controls worked. Despite this, it seems that some of the negative controls for the DNA-metabarcoding were slightly contaminated, but it had no effect on the final result. To limit uncertainty for qPCR results, a criterion was set defining lamprey presence if 2 out of 3 results showed presence of lamprey DNA. These criteria make it less probable that the results are false positive or false negative. However, thresholds affect detection (Harper et al., 2018), so my study might have had a too low or too high of a threshold.

I sampled in June, when lamprey are known to be active in Norway (Schartum & Kraabøl, 2013), so eDNA concentration should have be relatively high compared to other seasons. However, it might have been good to sample in a season with low water as this is shown to increase detection of eDNA (Bylemans et al., 2019).

A central part of this study was to compare the two eDNA methods, qPCR and DNA-metabarcoding, and an essential part of that comparison is the cost. The collection kits and lab analyses for qPCR was NOK 1500 per kit/filter. Thus for 2 filters per location, it was NOK 3000 per location. The cost applied to one genetic marker (one species). An additional cost of

NOK 700 could be added per species per filter if it was wanted to test for several species (such as if test for sea trout in addition to river trout). DNA – metabarcoding was a little bit more expensive. It was NOK 3500 per kit/filter, including collection kits (field equipment). Thus for 2 filters per location, it was NOK 7000 per location with 2 filters.

An added benefit to DNA-metabarcoding, is that it can investigate fish species diversity within a single sample with MiFish (Bessey et al., 2020; Ruppert et al., 2019). Although it is not a focus in my study, multiple species were found at each location (see Appendix table 3). My study had no need for the additional findings of other fish species, but the findings are beneficial for further research and management of Haldenvassdraget. An example of a species I found, in addition to, is eel, which is a species of interest in Haldenvassdraget.

Some of the biggest hinders in capturing optimal eDNA samples were the widths of the rivers, water volume and the lack of points with high turbulence that would have mixed the water well enough to get a good representation of the biodiversity. The uncertainty around how far eDNA can reliably be detected is also a hindrance. That being said it would seem that lamprey eDNA do not travel extreme distances (130 km) in Haldenvassdraget. If that was the case, I would have detected much more lamprey eDNA in the main river throughout the system.

## 4.2 Added knowledge to the distribution of lamprey in Haldenvassdraget

In addition to comparing the two eDNA methods, my study has added to the knowledge of distribution of river and brook lamprey in Haldenvassdraget. A sample absent of the targeted species eDNA, in this case lamprey eDNA, is not a guaranteed lack of presence of the targeted species at sampling site. Just because I did not find lamprey at a location, does not mean they were not there. This is supported by cross-referring my result with prior observations. Lamprey absence in samples does not prove actual absence (Roussel et al., 2015).

Out of the 45 locations I sampled from, 10 of them overlapped with previously reported observations of lamprey (Figure 3 C and D). Some of the locations do not have enough detail about exact placement making it difficult to find the exact spot the observation was made, so

some of the observation points on the map are estimates based on my knowledge of Haldenvassdraget. I was able to confirm presence of lamprey in Maltjennbekken (5), Hemneselva (7), Lowe (8), Upper Hafsteinselva (10) and Rødselva (40). There was also an observation from Mjerma where I sampled, I assumed it was around Lower Mjerma (15), due to Lundefossen separating Upper Mjerma (14) and Setten from the lower parts of Mjerma. I was however not able to confirm the presence of lamprey in Ulviksjøen (1), Ørje (22), and Mellebyelva (39) even though lamprey has been observed at some point. The observation Ulviksjøen (1) is from 1995 so it might be possible that the lamprey population is no longer there, if that is the case they probably moved, which lamprey are known to do (Dekker). However, it is also probable that I just did not capture any lamprey eDNA when I was sampling. For Ørje (22), presence has been confirmed for both river and brook lamprey from 2010 (2014), so it is most likely that I did not capture any eDNA. Considering the width of the river it was not feasible to acquire a well-mixed water sample. Presence of lamprey in Mellebyelva (39) was confirmed in 2015, and, again, the lamprey populations might have moved, or the more likely scenario, I just did not capture any eDNA. With my study results and previously published observations there is a total of 35 sightings of lamprey in Haldenvassdraget, with the majority of lamprey residing in the upper parts of the system (Figure 2 C and D).

## 4.3 Conclusions

eDNA methods can be better than traditional sampling according to some studies (Li et al., 2021; McColl-Gausden et al., 2021). It is however important to view eDNA results to be viewed in light of local knowledge, to hinder that wrong species being identified (Gargan et al., 2022). Even with the shortcoming of eDNA-metabarcoding, the consensus is that it is a good monitoring tool (Fujii et al., 2019; Gargan et al., 2022; Hänfling et al., 2016),

Both qPCR and DNA-metabarcoding worked as a tool to describe the distribution of river and brook lamprey in Haldenvassdraget. However, uncertainties of qPCR analyses failing or not, leads to inconclusive comparisons of detection probability, with qPCR potentially being statistically significantly more sensitive than DNA-metabarcoding.

My study suggests that DNA-metabarcoding is an acceptable monitoring tool for lamprey distribution, given the benefits it gives of a broader understanding of local biodiversity, and cost effectiveness as compared to qPCR.

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# 6 Appendixes

Appendix table 1 List of study sites in Haldenvassdraget. The volume of water (L) sampled for replicate A and B, and water temperature (°C) is given

| Sample ID | Date | Time | Locality | Latitude | Longitude | Water volume | Water temperature |
|---|---|---|---|---|---|---|---|
| 01A | 07.06.2021 | 09:30 | Ulviksjøen | 59.966797 | 11.495812 | 2 | 20 |
| 01B | 07.06.2021 | 09:30 | Ulviksjøen | 59.966797 | 11.495812 | 2.5 | 20 |
| 02A | 07.06.2021 | 10:50 | Eidsbekken | 59.884123 | 11.580333 | 2 | 20.8 |
| 02B | 07.06.2021 | 10:50 | Eidsbekken | 59.884123 | 11.580333 | 3 | 20.8 |
| 03A | 07.06.2021 | 11:30 | Lierelva | 59.886227 | 11.575236 | 1 | 20.5 |
| 03B | 07.06.2021 | 11:30 | Lierelva | 59.886227 | 11.575236 | 1 | 20.5 |
| 04A | 07.06.2021 | 12:10 | Elv fra Snartjern | 59.840777 | 11.538131 | 1 | 16.4 |
| 04B | 07.06.2021 | 12:10 | Elv fra Snartjern | 59.840777 | 11.538131 | 1 | 16.4 |
| 05A | 07.06.2021 | 13:25 | Malttjernbekken | 59.836745 | 11.479805 | 1.5 | 15.2 |
| 05B | 07.06.2021 | 13:25 | Malttjernbekken | 59.836745 | 11.479805 | 2 | 15.2 |
| 06A | 07.06.2021 | 14:00 | Dalselva | 59.727686 | 11.404894 | 1.5 | 15 |
| 06B | 07.06.2021 | 14:00 | Dalselva | 59.727686 | 11.404894 | 1 | 15 |
| 07A | 07.06.2021 | 14:45 | Hemneselva | 59.725505 | 11.420211 | 3 | 21.5 |
| 07B | 07.06.2021 | 14:45 | Hemneselva | 59.725505 | 11.420211 | 2 | 21.5 |
| 08A | 07.06.2021 | 15:40 | Lower Hafsteinselva | 59.722643 | 11.466323 | 2 | 21 |
| 08B | 07.06.2021 | 15:40 | Lower Hafsteinselva | 59.722643 | 11.466323 | 1 | 21 |
| 09A | 07.06.2021 | 16:15 | Hølansaelva | 59.720461 | 11.492098 | 1.5 | 20.3 |
| 09B | 07.06.2021 | 16:15 | Hølansaelva | 59.720461 | 11.492098 | 1 | 20.3 |
| 10A | 07.06.2021 | 16:50 | Upper Hafsteinselva | 59.747636 | 11.435987 | 1 | 17 |
| 10B | 07.06.2021 | 16:50 | Upper Hafsteinselva | 59.747636 | 11.435987 | 1 | 17 |
| 11A | 08.06.2021 | 09:50 | Setta | 59.84663 | 11.675412 | 1 | 16.5 |
| 11B | 08.06.2021 | 09:50 | Setta | 59.84663 | 11.675412 | 1 | 16.5 |
| 12A | 08.06.2021 | 10:45 | Langebruslora | 59.787913 | 11.726863 | 2 | 19 |
| 12B | 08.06.2021 | 10:45 | Langebruslora | 59.787913 | 11.726863 | 2 | 19 |
| 13A | 08.06.2021 | 11:45 | Langtjen | 59.810876 | 11.61592 | 5 | 19 |
| 13B | 08.06.2021 | 11:45 | Langtjen | 59.810876 | 11.61592 | 4 | 19 |
| 14A | 08.06.2021 | 13:40 | Upper Mjerma | 59.713321 | 11.555722 | 4 | 18.2 |
| 14B | 08.06.2021 | 13:40 | Upper Mjerma | 59.713321 | 11.555722 | 4.00 | 18.20 |
| 15A | 08.06.2021 | 14:30 | Lower Mjerma | 59.694242 | 11.528943 | 4 | 18.2 |
| 15B | 08.06.2021 | 14:30 | Lower Mjerma | 59.694242 | 11.528943 | 3 | 18.2 |
| 16A | 08.06.2021 | 16:45 | Nautebrofoss | 59.618259 | 11.596371 | 1.25 | 17.9 |
| 16B | 08.06.2021 | 16:45 | Nautebrofoss | 59.618259 | 11.596371 | 2 | 17.9 |
| 17A | 09.06.2021 | 13:00 | Østenbyelva | 59.533827 | 11.60172 | 1.5 | 19.2 |
| 17B | 09.06.2021 | 13:00 | Østenbyelva | 59.533827 | 11.60172 | 2 | 19.2 |
| 18A | 09.06.2021 | 13:45 | Taraldruelva | 59.581574 | 11.565786 | 1.5 | 15.6 |
| 18B | 09.06.2021 | 13:45 | Taraldruelva | 59.581574 | 11.565786 | 2 | 15.6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 19A | 09.06.2021 | 14:30 | Åsebyelva | 59.600175 | 11.61974 | 1.5 | 17 |
| 19B | 09.06.2021 | 14:30 | Åsebyelva | 59.600175 | 11.61974 | 1 | 17 |
| 20A | 09.06.2021 | 15:15 | Langnes | 59.546237 | 11.626861 | 2 | 17.2 |
| 20B | 09.06.2021 | 15:15 | Langnes | 59.546237 | 11.626861 | 2.5 | 17.2 |
| 21A | 09.06.2021 | 16:00 | Engerelva | 59.491057 | 11.667821 | 1.5 | 17.8 |
| 21B | 09.06.2021 | 16:00 | Engerelva | 59.491057 | 11.667821 | 1.5 | 17.8 |
| 22A | 10.06.2021 | 08:30 | Ørje | 59.472415 | 11.654516 | 2.5 | 13.7 |
| 22B | 10.06.2021 | 08:30 | Ørje | 59.472415 | 11.654516 | 3 | 13.7 |
| 23A | 10.06.2021 | 09:20 | Bøenselva | 59.461618 | 11.660805 | 2 | 18.4 |
| 23B | 10.06.2021 | 09:20 | Bøenselva | 59.461618 | 11.660805 | 2 | 18.4 |
| 24A | 10.06.2021 | 09:50 | Gunnengbekken | 59.366216 | 11.689881 | 1 | 14.3 |
| 24B | 10.06.2021 | 09:50 | Gunnengbekken | 59.366216 | 11.689881 | 1 | 14.3 |
| 25A | 10.06.2021 | 10:40 | Skinnarbutjenn | 59.351489 | 11.733146 | 3 | 19.2 |
| 25B | 10.06.2021 | 10:40 | Skinnarbutjenn | 59.351489 | 11.733146 | 2.5 | 19.2 |
| 26A | 10.06.2021 | 11:20 | Langetjernelva | 59.329994 | 11.685982 | 2 | 18.3 |
| 26B | 10.06.2021 | 11:20 | Langetjernelva | 59.329994 | 11.685982 | 2 | 18.3 |
| 27A | 10.06.2021 | 12:00 | Fangebekken | 59.288988 | 11.678935 | 1 | 14.9 |
| 27B | 10.06.2021 | 12:00 | Fangebekken | 59.288988 | 11.678935 | 1 | 14.9 |
| 28A | 10.06.2021 | 12:40 | Fossby | 59.220603 | 11.6962 | 1 | 16.3 |
| 28B | 10.06.2021 | 12:40 | Fossby | 59.220603 | 11.6962 | 1 | 16.3 |
| 29A | 10.06.2021 | 13:20 | Skolleborg | 59.232771 | 11.671846 | 1.5 | 18.7 |
| 29B | 10.06.2021 | 13:20 | Skolleborg | 59.232771 | 11.671846 | 1 | 18.7 |
| 30A | 10.06.2021 | 14:00 | Verksbrua | 59.25494 | 11.64334 | 2.5 | 20.1 |
| 30B | 10.06.2021 | 14:00 | Verksbrua | 59.25494 | 11.64334 | 2.5 | 20.1 |
| 31A | 10.06.2021 | 14:30 | Tenebekken | 59.280254 | 11.65288 | 1 | 15.3 |
| 31B | 10.06.2021 | 14:30 | Tenebekken | 59.280254 | 11.65288 | 1 | 15.3 |
| 32A | 10.06.2021 | 15:20 | Strømsfoss | 59.301061 | 11.658639 | 3 | 16.6 |
| 32B | 10.06.2021 | 15:20 | Strømsfoss | 59.301061 | 11.658639 | 3 | 16.6 |
| 33A | 11.06.2021 | 08:30 | Lielva | 59.461153 | 11.644340 | 1.5 | 18.8 |
| 33B | 11.06.2021 | 08:30 | Lielva | 59.461153 | 11.644340 | 1.5 | 18.8 |
| 34A | 11.06.2021 | 09:10 | Valbyelva | 59.381098 | 11.642453 | 1 | 14.5 |
| 34B | 11.06.2021 | 09:10 | Valbyelva | 59.381098 | 11.642453 | 1 | 14.5 |
| 35A | 11.06.2021 | 10:15 | Holmegilelva | 59.142629 | 11.739045 | 4 | 20.8 |
| 35B | 11.06.2021 | 10:15 | Holmegilelva | 59.142629 | 11.739045 | 4 | 20.8 |
| 36A | 11.06.2021 | 11:20 | Kverntjern (Remne) | 59.140828 | 11.660145 | 2 | 20.5 |
| 36B | 11.06.2021 | 11:20 | Kverntjern (Remne) | 59.140828 | 11.660145 | 2 | 20.5 |
| 37A | 11.06.2021 | 12:10 | Stenselva | 59.14554 | 11.638203 | 3.5 | 15.5 |
| 37B | 11.06.2021 | 12:10 | Stenselva | 59.14554 | 11.638203 | 3 | 15.5 |
| 38A | 11.06.2021 | 13:00 | Ganerødelva | 59.144157 | 11.534509 | 2.33 | 20.4 |
| 38B | 11.06.2021 | 13:00 | Ganerødelva | 59.144157 | 11.534509 | 2.2 | 20.4 |
| 39A | 11.06.2021 | 14:10 | Mellebyelva | 59.180468 | 11.689811 | 1 | 20.1 |
| 39B | 11.06.2021 | 14:10 | Mellebyelva | 59.180468 | 11.689811 | 1 | 20.1 |
| 40A | 13.06.2021 | 14:10 | Rødselva | 59.171888 | 11.545509 | 1 | 20.2 |
| 40B | 13.06.2021 | 14:10 | Rødselva | 59.171888 | 11.545509 | 1 | 20.2 |
| 41A | 13.06.2021 | 14:50 | Rjørelva | 59.183022 | 11.51762 | 1.5 | 21.3 |
| 41B | 13.06.2021 | 14:50 | Rjørelva | 59.183022 | 11.51762 | 1.5 | 21.3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 42A | 13.06.2021 | 15:30 | Asakbekken | 59.138606 | 11.451391 | 1 | 13.7 |
| 42B | 13.06.2021 | 15:30 | Asakbekken | 59.138606 | 11.451391 | 1 | 13.7 |
| 43A | 13.06.2021 | 16:30 | Tistedalsfoss | 59.130336 | 11.437732 | 4 | 15.3 |
| 43B | 13.06.2021 | 16:30 | Tistedalsfoss | 59.130336 | 11.437732 | 4 | 15.3 |
| 44A | 13.06.2021 | 18:00 | Skåningsfoss | 59.125933 | 11.416768 | 5 | 21 |
| 44B | 13.06.2021 | 18:00 | Skåningsfoss | 59.125933 | 11.416768 | 5 | 21 |
| 45A | 13.06.2021 | 20:00 | Porsnes sluser | 59.123438 | 11.39958 | 4 | 20.5 |
| 45B | 13.06.2021 | 20:00 | Porsnes sluser | 59.123438 | 11.39958 | 4 | 20.5 |

Appendix table 2 Optical density (OD) showing DNA-quantity and quality of the DNA-extracts.

| Sample ID | OD | 260-280 | 260-230 |
|---|---|---|---|
| 01A | 67.17 | 1.57 | 1.16 |
| 01B | 84.79 | 1.63 | 1.15 |
| 02A | 154.30 | 1.62 | 0.91 |
| 02B | 168.60 | 1.67 | 0.94 |
| 03A | 354.90 | 1.73 | 1.26 |
| 03B | 345.80 | 1.70 | 1.19 |
| 04A | 177.40 | 1.59 | 0.93 |
| 04B | 161.10 | 1.56 | 0.87 |
| 05A | 92.13 | 1.65 | 0.95 |
| 05B | 115.80 | 1.69 | 1.09 |
| 06A | 308.00 | 1.53 | 0.90 |
| 06B | 250.90 | 1.70 | 1.05 |
| 07A | 266.60 | 1.94 | 1.73 |
| 07B | 193.20 | 1.93 | 1.63 |
| 08A | 268.40 | 1.61 | 1.02 |
| 08B | 275.70 | 1.83 | 1.38 |
| 09A | 327.50 | 1.74 | 1.23 |
| 09B | 232.60 | 1.84 | 1.47 |
| 10A | 312.80 | 1.63 | 1.00 |
| 10B | 328.50 | 1.68 | 1.12 |
| 11A | 117.90 | 1.68 | 1.13 |
| 11B | 45.57 | 1.51 | 1.07 |
| 12A | 96.90 | 1.74 | 1.33 |
| 12B | 90.04 | 1.70 | 1.17 |
| 13A | 242.20 | 1.76 | 1.18 |
| 13B | 84.02 | 1.60 | 1.10 |
| 14A | 224.20 | 1.63 | 0.99 |
| 14B | 65.22 | 1.70 | 0.91 |
| 15A | 283.30 | 1.72 | 1.18 |
| 15B | 133.50 | 1.67 | 1.07 |
| 16A | 171.50 | 1.60 | 1.00 |
| 16B | 141.90 | 1.61 | 0.99 |
| 17A | 105.30 | 1.78 | 1.43 |

| | | | |
|---|---|---|---|
| 17B | 187.40 | 1.87 | 1.50 |
| 18A | 895.30 | 1.90 | 1.31 |
| 18B | 494.80 | 1.78 | 1.31 |
| 19A | 23.79 | 1.87 | 6.00 |
| 19B | 67.37 | 1.70 | 1.57 |
| 20A | 250.40 | 1.70 | 1.13 |
| 20B | 214.60 | 1.70 | 1.11 |
| 21A | 102.00 | 1.65 | 1.17 |
| 21B | 135.70 | 1.82 | 1.43 |
| 22A | 418.20 | 1.72 | 1.29 |
| 22B | 421.50 | 1.79 | 1.39 |
| 23A | 423.20 | 1.60 | 0.94 |
| 23B | 387.40 | 1.65 | 0.92 |
| 24A | 284.00 | 1.86 | 1.52 |
| 24B | 247.80 | 1.78 | 1.32 |
| 25A | 342.30 | 1.80 | 1.38 |
| 25B | 222.40 | 1.71 | 1.15 |
| 26A | 135.80 | 1.78 | 1.38 |
| 26B | 129.90 | 1.86 | 1.48 |
| 27A | 102.80 | 1.68 | 1.35 |
| 27B | 96.26 | 1.62 | 1.28 |
| 28A | 177.10 | 1.60 | 0.84 |
| 28B | 58.52 | 1.44 | 0.64 |
| 29A | 129.9 | 1.72 | 1.17 |
| 29B | 91.32 | 1.69 | 1.17 |
| 30A | 43.7 | 1.47 | 0.7 |
| 30B | 45.2 | 1.65 | 1.32 |
| 31A | 429.6 | 1.36 | 0.86 |
| 31B | 160 | 1.79 | 1.23 |
| 32A | 548.5 | 1.71 | 1.37 |
| 32B | 499.1 | 1.76 | 1.49 |
| 33A | 78.36 | 1.73 | 1.38 |
| 33B | 125.7 | 1.78 | 1.41 |
| 34A | 277.3 | 1.82 | 1.36 |
| 34B | 156.4 | 1.8 | 1.35 |
| 35A | 70.06 | 1.4 | 0.58 |
| 35B | 76.13 | 1.45 | 0.57 |
| 36A | 173.3 | 1.62 | 0.96 |
| 36B | 97.32 | 1.51 | 0.86 |
| 37A | 406.4 | 1.74 | 1.33 |
| 37B | 240.1 | 1.89 | 1.57 |
| 38A | 151.7 | 1.73 | 1.22 |
| 38B | 101.9 | 1.76 | 1.59 |
| 39A | 316.2 | 1.63 | 0.91 |
| 39B | 153.3 | 1.39 | 0.51 |
| 40A | 77.62 | 1.72 | 1.27 |

| | | | |
|---|---|---|---|
| 40B | 238.3 | 1.9 | 1.75 |
| 41A | 543.8 | 1.65 | 1.04 |
| 41B | 687.4 | 1.71 | 0.96 |
| 42A | 367.4 | 1.96 | 1.68 |
| 42B | 298.9 | 1.97 | 1.73 |
| 43A | 173.6 | 1.78 | 1.35 |
| 43B | 375.3 | 1.46 | 0.97 |
| 44A | 242.7 | 1.55 | 0.92 |
| 44B | 566.9 | 1.69 | 1.41 |
| 45A | 394.5 | 1.85 | 1.57 |
| 45B | 544.1 | 1.71 | 1.4 |

Appendix table 3 No of reads in the different filtering steps as part of the bioinformatic pipeline.

| PrøveID | raw | input | filtered | denoisedF | denoisedR | merged | nonchim | pct_retained |
|---|---|---|---|---|---|---|---|---|
| 01A | 1455086 | 1062405 | 948304 | 946120 | 945593 | 933420 | 921167 | 63.31 |
| 01B | 1323113 | 935574 | 856976 | 854968 | 854182 | 843781 | 832564 | 62.92 |
| 02A | 1657246 | 1294966 | 1150916 | 1148201 | 1148250 | 1129475 | 1099906 | 66.37 |
| 02B | 1583681 | 1239705 | 1114008 | 1111423 | 1110675 | 1087359 | 1050906 | 66.36 |
| 03A | 1158269 | 824013 | 743885 | 742136 | 741844 | 732210 | 722124 | 62.35 |
| 03B | 1235933 | 878380 | 777985 | 775945 | 775860 | 765656 | 753274 | 60.95 |
| 04A | 1646107 | 1380993 | 1261281 | 1256340 | 1255571 | 1221950 | 1183165 | 71.88 |
| 04B | 1580171 | 1353233 | 1227704 | 1222223 | 1221426 | 1183311 | 1136069 | 71.90 |
| 05A | 953837 | 691860 | 588129 | 586627 | 586214 | 577255 | 568899 | 59.64 |
| 05B | 1156764 | 876671 | 793951 | 792015 | 791769 | 780411 | 767953 | 66.39 |
| 06A | 790254 | 481334 | 434460 | 434177 | 433991 | 431932 | 426385 | 53.96 |
| 06B | 867029 | 380806 | 338488 | 338200 | 338224 | 334813 | 332007 | 38.29 |
| 07A | 1009808 | 683317 | 505775 | 505035 | 505062 | 501577 | 496395 | 49.16 |
| 07B | 807399 | 479007 | 434217 | 433858 | 433892 | 431838 | 429060 | 53.14 |
| 08A | 429139 | 263564 | 239376 | 239135 | 239014 | 235784 | 231809 | 54.02 |
| 08B | 374327 | 213285 | 194728 | 194211 | 194017 | 191270 | 187355 | 50.05 |
| 09A | 1308500 | 918702 | 785450 | 783602 | 783243 | 774497 | 764131 | 58.40 |
| 09B | 820822 | 441237 | 391429 | 391044 | 391066 | 389560 | 386818 | 47.13 |
| 10A | 1379215 | 1034237 | 936521 | 934166 | 934210 | 913186 | 898145 | 65.12 |
| 10B | 971990 | 738383 | 653938 | 652063 | 651668 | 636529 | 624973 | 64.30 |
| 11A | 1227277 | 845897 | 761377 | 759424 | 759014 | 743635 | 729806 | 59.47 |
| 11B | 1193528 | 225942 | 177654 | 177533 | 177543 | 177015 | 176399 | 14.78 |
| 12A | 1182251 | 748677 | 678988 | 677972 | 677351 | 670813 | 665332 | 56.28 |
| 12B | 1135518 | 704805 | 626534 | 625411 | 625283 | 618903 | 611714 | 53.87 |
| 13A | 1139099 | 742628 | 446291 | 445760 | 444883 | 441420 | 436388 | 38.31 |
| 13B | 1299729 | 841699 | 756843 | 755174 | 754793 | 746359 | 739882 | 56.93 |
| 14A | 1270575 | 487565 | 401367 | 401123 | 401227 | 399924 | 399600 | 31.45 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14B | 496158 | 370678 | 307604 | 307571 | 307534 | 305114 | 304399 | 61.35 |
| 15A | 998805 | 733936 | 486371 | 485622 | 485664 | 481865 | 476432 | 47.70 |
| 15B | 537641 | 382722 | 338714 | 338054 | 337795 | 334494 | 331098 | 61.58 |
| 16A | 398782 | 264403 | 224284 | 223832 | 223847 | 221247 | 218321 | 54.75 |
| 16B | 545654 | 418196 | 377990 | 376844 | 376760 | 370862 | 365093 | 66.91 |
| 17A | 904553 | 536732 | 379727 | 379624 | 379553 | 378345 | 377946 | 41.78 |
| 17B | 848947 | 424423 | 376652 | 376450 | 376329 | 373886 | 372382 | 43.86 |
| 18A | 1848867 | 1439197 | 1293461 | 1290026 | 1290000 | 1265128 | 1234862 | 66.79 |
| 18B | 2186670 | 1752046 | 1613300 | 1608670 | 1608115 | 1568269 | 1519424 | 69.49 |
| 19A | 1494406 | 1195186 | 1088603 | 1083408 | 1083047 | 1049092 | 1016677 | 68.03 |
| 19B | 1225045 | 951403 | 793783 | 791652 | 790931 | 774680 | 758840 | 61.94 |
| 20A | 1123666 | 917989 | 777969 | 774809 | 773949 | 754933 | 741848 | 66.02 |
| 20B | 1137157 | 905002 | 832951 | 829918 | 829179 | 811403 | 795849 | 69.99 |
| 21A | 1531607 | 1191316 | 1098824 | 1094632 | 1094704 | 1073616 | 1053905 | 68.81 |
| 21B | 2261556 | 1814918 | 1538957 | 1535452 | 1534625 | 1508322 | 1483242 | 65.59 |
| 22A | 774687 | 563375 | 508850 | 507789 | 507515 | 502850 | 496382 | 64.08 |
| 22B | 939129 | 697651 | 608640 | 607622 | 607253 | 601536 | 594419 | 63.29 |
| 23A | 578616 | 417497 | 372675 | 371593 | 371550 | 367681 | 361511 | 62.48 |
| 23B | 396444 | 303459 | 265098 | 264033 | 263961 | 260242 | 257687 | 65.00 |
| 24A | 1305015 | 1028230 | 903256 | 899828 | 899393 | 876781 | 856967 | 65.67 |
| 24B | 1756760 | 1371703 | 1260156 | 1256162 | 1255146 | 1223837 | 1190277 | 67.75 |
| 25A | 1218850 | 857455 | 684772 | 683594 | 683533 | 677963 | 669556 | 54.93 |
| 25B | 1242227 | 855140 | 778782 | 776714 | 776227 | 766422 | 754691 | 60.75 |
| 26A | 1237381 | 735624 | 480504 | 480228 | 480196 | 477621 | 470957 | 38.06 |
| 26B | 1447843 | 1135961 | 1023593 | 1019834 | 1019139 | 998772 | 981783 | 67.81 |
| 27A | 1130362 | 943496 | 840522 | 837904 | 837069 | 820089 | 803981 | 71.13 |
| 27B | 1070393 | 911079 | 726092 | 723710 | 723721 | 708962 | 696787 | 65.10 |
| 28A | 973822 | 824078 | 640042 | 638869 | 638596 | 629680 | 618090 | 63.47 |
| 28B | 483830 | 225461 | 37467 | 37440 | 37383 | 37261 | 37256 | 7.70 |
| 29A | 795269 | 598800 | 446351 | 445754 | 445700 | 439883 | 432938 | 54.44 |
| 29B | 1109002 | 838214 | 757921 | 756784 | 755896 | 747483 | 738364 | 66.58 |
| 30A | 686691 | 494429 | 229701 | 229655 | 229609 | 229161 | 228859 | 33.33 |
| 30B | 2238568 | 1606631 | 1448081 | 1444321 | 1443283 | 1418370 | 1387461 | 61.98 |
| 31A | 382723 | 290621 | 106369 | 106254 | 106321 | 106062 | 106060 | 27.71 |
| 31B | 817229 | 632536 | 551571 | 549445 | 549028 | 536695 | 525289 | 64.28 |
| 32A | 1240336 | 868960 | 776394 | 775185 | 774794 | 768794 | 757618 | 61.08 |
| 32B | 1007850 | 720262 | 662772 | 661753 | 661579 | 657486 | 652457 | 64.74 |
| 33A | 1209018 | 952436 | 869812 | 866422 | 866251 | 845386 | 827429 | 68.44 |
| 33B | 1205502 | 760866 | 683414 | 682511 | 682696 | 676816 | 658954 | 54.66 |
| 34A | 1634446 | 1319000 | 1110080 | 1107260 | 1106883 | 1086620 | 1064472 | 65.13 |
| 34B | 1171357 | 750115 | 497545 | 497023 | 496976 | 494541 | 491411 | 41.95 |
| 35A | 38970 | 33375 | 10632 | 10609 | 10621 | 10552 | 10551 | 27.07 |
| 35B | 416987 | 172526 | 118477 | 118462 | 118433 | 118153 | 118090 | 28.32 |
| 36A | 765411 | 526251 | 480079 | 479938 | 479933 | 478764 | 471625 | 61.62 |
| 36B | 62624 | 59253 | 5 | 2 | 2 | 2 | 2 | 0.00 |
| 37A | 1544958 | 1027355 | 931407 | 930223 | 930142 | 923167 | 908661 | 58.81 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 37B | 766127 | 538888 | 212066 | 211860 | 211843 | 211003 | 209867 | 27.39 |
| 38A | 793794 | 584364 | 523985 | 523753 | 523254 | 519814 | 518056 | 65.26 |
| 38B | 736857 | 580872 | 516957 | 516259 | 516227 | 512539 | 508283 | 68.98 |
| 39A | 914209 | 673467 | 601978 | 601683 | 601638 | 597243 | 592529 | 64.81 |
| 39B | 216904 | 122869 | 59517 | 59497 | 59486 | 59383 | 59377 | 27.37 |
| 40A | 168033 | 100918 | 41722 | 41652 | 41654 | 41543 | 41535 | 24.72 |
| 40B | 1303004 | 869905 | 794138 | 792251 | 791838 | 780668 | 767773 | 58.92 |
| 41A | 1002971 | 675493 | 614587 | 614256 | 614357 | 611668 | 605568 | 60.38 |
| 41B | 909027 | 688181 | 511351 | 511059 | 510798 | 508640 | 503175 | 55.35 |
| 42A | 1541405 | 1192740 | 1095746 | 1092988 | 1091944 | 1068436 | 1042102 | 67.61 |
| 42B | 1472582 | 1142650 | 1037900 | 1033887 | 1032775 | 998808 | 969002 | 65.80 |
| 43A | 937270 | 576230 | 515045 | 513988 | 513602 | 508837 | 502874 | 53.65 |
| 43B | 1402609 | 749325 | 682932 | 681878 | 681727 | 675816 | 670631 | 47.81 |
| 44A | 1038517 | 426209 | 369992 | 369682 | 369601 | 367050 | 365709 | 35.21 |
| 44B | 1010522 | 579909 | 514339 | 513362 | 512757 | 509963 | 505451 | 50.02 |
| 45A | 996399 | 612092 | 559950 | 558916 | 558441 | 554389 | 550469 | 55.25 |
| 45B | 431615 | 254855 | 228299 | 227733 | 227595 | 226173 | 224766 | 52.08 |
| NEG_150721 | 25190 | 9898 | 45 | 45 | 43 | 43 | 43 | 0.17 |
| NEG_220721 | 123358 | 105000 | 43 | 43 | 43 | 43 | 43 | 0.03 |
| NEG1 | 56199 | 36257 | 7901 | 7894 | 7894 | 7894 | 7841 | 13.95 |
| NEG2 | 139959 | 112760 | 1818 | 1812 | 1812 | 1812 | 1809 | 1.29 |
| NEG3 | 31462 | 17151 | 0 | NA | NA | NA | NA | NA |

Appendix table 4 DNA-metabarcoding results. All fish species found in the different samling location. With the species eDNA abundance.