Environmental DNA methods for detection of wild Atlantic salmon (*Salmo salar L.*) in Haugsdalselva, Masfjorden, Norway º

Rachel Rodrigues Lima

Master's Thesis

Molecular Ecology Group

Department of Biological Sciences

University of Bergen and Norwegian Research Centre AS

2022

Scientific Environment

This thesis is the result of the specialisation in Biodiversity, evolution, and ecology in the Master´s Programme in Biology in the University of Bergen. The work present in this thesis was carried out with the Molecular Ecology and Paleogenomics group (MEP) from the Norwegian Research Centre.
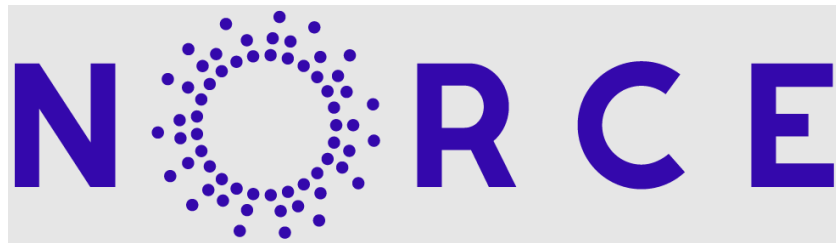
1. Abstract

Atlantic salmon is of worldwide importance and is one of the many other freshwater species experiencing rapid decline, despite multiple protection efforts. Accurate biodiversity data, distribution patterns and stock assessments are key for successful conservation and molecular methods used for detection of genetic material shed by organisms in the environment have been successfully applied to several species and various aquatic systems. Environmental DNA (eDNA) methodologies have been proposed as a promising non-invasive and less labour intensive and time-consuming alternative or supplementary tool for biomonitoring.

The purpose of this thesis was to explore and compare different molecular protocols for detection of Atlantic Salmon DNA. A comparison between different filter pore sizes, DNA extraction kits and PCR machinery and chemistries to maximize eDNA detection in freshwater. DNeasy Power Water kit had the best performance when evaluating for total DNA yield, removal of inhibitors and detection of Atlantic salmon. The probe assay was more sensitive than SYBR assay. Both qPCR and ddPCR were able to detect Atlantic Salmon during the year but ddPCR performed better than qPCR in the Summer. Overall, techniques used successfully detected the presence of Atlantic salmon showed consistent with previous assessments of salmon presence in Haugsdalselva.

Keywords: Salmo salar L., eDNA, biomonitoring, method comparison, western Norway, fresh water

2. Table of Contents

3. Introduction

3.1 Wild Atlantic salmon

Atlantic salmon (*Salmo salar L.*) is a fish species that belongs to the Salmonidae family (salmonids), which include the genera Salmo (salmon, trout), Salvelinus (char), Oncorhynchus (pacific salmon), Coregonus (whitefishes) and Thymallus (graylings). Salmonids show a laterally compressed body form and dorsal adipose fin, posterior to the main dorsal fin. The specific name of Atlantic salmon, *salar*, means leaper, probably being given due to their ability of leap up high waterfalls on its return during spawning migration (Øystein Aas et al., 2010).

Atlantic salmon occurs in temperate waters and subarctic regions of the North Atlantic Ocean but like most Salmonids, they have big differences in life-history within and between locations or populations. They can occupy a wide variety of landscapes and climates and can move between fresh and salt water, behaviour known as anadromy. They present more than 2000 genetically distinct populations (Gillson et al., 2022) from which most are anadromous and represent the archetypal Atlantic salmon. Populations can also show behaviours of non-anadromy or partial anadromy, meaning some spend their whole life cycle in fresh water while others can be prevented by low sea temperatures in some years to migrate extensive distances and spent their marine phase in estuaries (Webb et al., 2007).

Spawning takes place over a period of weeks between autumn, winter, in deep and fast flowing waters to early spring. Although the timing of can vary depending on latitude, in most populations spawning occurs over a period of weeks between October and December. They spawn in fresh water, the eggs hatch mostly during spring and remain in fresh waters through the juvenile, alevins, fry and parr phases. The next stage, smolt, is associated with the migration to the ocean (Webb et al., 2007).

This species is capable of as long as 2000 km migration in the open ocean and to return to natal river for spawning (Jonsson & Jonsson, 2011b). The smolts usually migrate to sea in the spring to feed in the North Atlantic, and seldom return to fresh water the same year of sea migration. Reproductive migration is another factor that varies widely among populations, as some can take as little as 3-5 months (Webb et al., 2007) and others can take from 1 to 4 years before becoming mature enough to return to the home river. Salmon species can have more than one reproduction year but waiting usually around 2 years before spawning again. Few individuals spawn more than three times or live longer than 10 years. (Jonsson & Jonsson, 2011b).

3.2 Conservation – special focus on salmonids

Atlantic salmon is of social, cultural, and economic importance. It is used as a sentinel species for monitoring aquatic environment through ecotoxicological studies and has been the subject of a large research effort focusing on its biology, life history, population dynamics, biogeography, genetics, and phylogeny. No other group of fish species received as much attention from commercial and scientific efforts as salmonids (Davidson et al., 2010).

Present in more than 400 watercourses in Norway, they represent around 25% of the world´s healthy populations (Forseth et al., 2017), holding most of the European wild Atlantic salmon stock (Bolstad et al., 2017).

In Norway, salmon has traditionally been harvested in the sea as an important food source, but today recreational fishing in rivers that attracted anglers not only from Norway but also from thousands of foreign tourists, is more important than the direct commercial value of the fisheries (Liu et al., 2011).

Wild Atlantic salmon stocks have experienced a steep decline in the last few decades, mainly associated anthropogenic impacts as overexploitation (overfishing) and habitat destructions. Rivers have been negatively affected by hydropower production due to river regulation, while salmon lice are considered as a major threat from aquaculture in the marine environment (see Forseth et al 2017).

Climate change has a negative impact mainly in the southern parts of the species distribution due temperature increase in fresh water, while in the ocean the impact is thought to be connected through food chain, the reduced abundance of keystone food organisms, resulting directly in decrease of Atlantic salmon abundance in the North Atlantic (Jonsson & Jonsson, 2011a). Due to salmon's phenotypic plasticity, populations responses to short term climate changes result in variations of their behaviour, physiology and life history, affecting directly spawning, fry emergence, rate of development and growth, age, size at migration, sexual maturation and life span (Jonsson & Jonsson, 2009).

In addition, the growth of fish farming industry has created problems for wild populations. Hatcheries and fish farms can spread pathogens to wild fish such as sea lice such as salmon louse (*Lepeophtheirus salmonis*) (Frazer, 2009). Cultured salmonids have been artificially selected to traits that improve growth rates and others of commercial interest, and interbreeding between escaped farmed salmon and wild populations can cause loss of genetic variation with a direct impact to populations local adaptations and ecological interactions. The introduction of foreign alleles is due to introgression(Roberge et al., 2008) (Forseth et al 2017), which has been detected in most of the Norwegian Salmon stock. (Bolstad et al., 2017) and has been suggested as one of the most significant threats to native *Salmon salar L.* populations where aquaculture is practiced. (Mahlum 2019).

To achieve proper conservation of wild Atlantic salmon, it is necessary to understand species distribution (Atkinson et al., 2018). While many studies have focused on the distribution in freshwater (S. K. Mahlum et al., 2018), there is little understanding of the marine phase of their life cycle (Øystein

Aas et al., 2010). Other challenges arise due to interactions between salmon populations and their environment (S. K. Mahlum et al., 2018), including changes in migration timing and different life-history patterns (Harvey et al., 2020), detection of species in different life stages, low density populations and difficult access in many environments (Dejean et al. 2011).

Generally, fish management and conservation are based on information collected with different survey techniques. Conventional techniques such as mark-recapture, seining, trapping, netting and electrofishing can be harmful to individuals at critical times in their life cycle, despite being invasive, can only be carried out in areas where conditions are favourable (Thomsen et al., 2012) and are more costly and are more time and labour intensive, mobilizing multiple fisheries professionals (Evans et al., 2017). Also, standard techniques for biomonitoring generate limited information (Baird, 2012), as it provides species-focused data which doesn't account for knowledge of ecosystems goods and services and can strongly influence policy development (Baird & Hajibabaei, 2012).

In Norway, electrofishing has been broadly used for surveillance and fish monitoring in rivers. Despite providing valuable information of population structure (Evans et al., 2017), electrofishing has a number of methodological difficulties. It can be hindered by logistical problems, such as presence of sites with high salinity levels, deep or turbid or in waterways with abundant large debris and fails to detect species in particular developmental stages (Ficetola et al., 2008; McColl-Gausden et al., 2021). Also, it requires heavy equipment and training besides posing harmful effects for fish species such as haemorrhages, spinal injuries, bleeding at gills and mortality by asphyxiation; specially problematic for rare or threatened species (Sigsgaard et al., 2015; Snyder, 2003).

Stock assessment has also used angling-based methods from commercial or recreational fisheries. In Norway, it is adopted for surveillance of escapees from fish farming. Pitfalls of this technique include problems with timing; fishing in Norway for salmon occurs during summer and a mismatch between sampling and fish presence for spawning due to variation in optimal river conditions for upstream migration causing a sampling bias or due to reduced catch after river entry. It can require extensive time, mobilize a great deal of personnel and equipment and are limited by impractical habitats or prohibited fishing areas (S. Mahlum et al., 2019).

Quantification and qualitative data on fish populations in streams can also be obtained from traps, counting fences and other installations that detect fish upstream migration, but those can be costly, difficult to operate and not feasible to use in different rivers (Skoglund et al., 2021).

Observational methods such as video surveillance, camera traps and drift diving are commonly used non-invasive techniques for visually identification of fish species, sex, size, and origin (farmed or wild salmonids) in accordance with standardised methods (NS 9456:2015). Since 2014, drift diving/snorkelling is one supplementary method that has been broadly used in Norwegian monitoring

programs of farmed and scaped salmon but despite being accurate and versatile, it is highly reliant on factors such as visual conditions underwater – turbidity and light conditions, the distance between the fish and observer, experience of the observer to able to observe each fish well enough to correctly distinguish escapees and wild fish (S. Mahlum et al., 2019; Skoglund et al., 2021).

An emergent alternative to those disadvantages is environmental DNA (eDNA) based methods. Surveillance based on detection of eDNA can be an alternative strategy to obtain information on species distribution, improving detection sensibility and efficiency (Evans et al., 2017), as it feasible in areas other methods can´t be used due to conditions of difficult to reach areas, private property, pollution, high flow, water depth, lower cost, for its capacity to detect cryptic species and finally its sensitivity and sampling ease allows rapid surveys in large geographic areas in comparison with other traditional methods (Evans et al., 2017; Sigsgaard et al., 2015).

Methodologies based on eDNA detection have been used in fresh-water and marine habitats across literature (Agersnap et al., 2017; Atkinson et al., 2018; Knudsen et al., 2019; Sigsgaard et al., 2015), can provide fast and reliable information (Deiner et al., 2021) and have been considered a promising source of ecological information for usage in conservation and biomonitoring in fisheries for its capacity of detecting invasive, rare, cryptic, threatened species in different developmental stages. In addition, it is less labour intensive, more sensitive than traditional sampling methods, has a reduced risk of unintentional secondary dispersal of alien species and diseases and is non-invasive, as no fish or other macroorganisms are caught, disturbed or killed during assessment, reduces the risk of unintentional secondary dispersal of alien species and diseases (Fossøy et al., 2020; Valentini et al., 2016).

3.3 Environmental DNA (eDNA)

Environmental DNA broadly refers to the remains of intracellular or extracellular DNA present and directly collected in environments such as soil, sediment, air and water that originates from tissues, faeces, urine, hair, scales and other biological materials (Kirtane et al., 2021; Stewart, 2019; Taberlet et al., 2012). Scales, skin, mucus or by excretory processes as urine and faecal matter are commonly the source of DNA shed by fish in the environment (Yates et al., 2021), and it can persist in the water column and be detectable for up to 60 days (Kelly et al., 2014; Rourke et al., 2022).

In conservation species distribution assessment requires primarily data on presence or absence of species, which eDNA has been proven to successfully provide even in cases of rare species that wouldn´t be detected and environments of difficult access that commonly can impact electrofishing, limiting information on presence of targeted species in those areas. (Evans et al., 2017; Sigsgaard et al., 2015; Atkinson et al., 2015). Additionally, environmental DNA can be used to retrieve using

metabarcoding and/or metagenomics approaches to identify and quantify different taxa even at population level (Xing et al., 2022; Kirtane et al., 2021).

Ecological studies using eDNA require three steps: a) sample collection and preservation, b) extraction and purification of DNA, c) detection of specific target DNA. For aquatic studies, water samples are obtained from lakes, rivers, streams, sea, artificial ponds/reservoirs/canals/ditches, lagoons or in experimental settings, such as water tanks. Challenges such as recovering sufficient eDNA yields and filtering enough samples are an important factor for detection of species. Volumes ranging from 15 mL to 45 L have been used for eDNA research, of which 1 to 2 L is most used in natural water bodies (Xing 2022). Sampling can be made using techniques such as filtration, precipitation, and centrifugation. Filtration is commonly used and consists of water samples being pumped through a filter. The filter material and pore size vary across literature, pore sizes range from 0.22 to 0.5 μm and filters can be of Glass Fiber, Polycarbonate, Nylon, Cellulose nitrate, polyether sulfone, polyvinylidene difluoride, mixed cellulose acetate and nitrate, and in format of a filter capsule; Sterivex enclosure filter. In the precipitation method, ethanol or isopropanol are added in samples with sodium acetate and is commonly used for small sample volumes. Centrifugation consists of centrifugation of water samples and subsequent discard of supernatant, this method is not commonly used for water samples as it´s not suitable for large volumes of water, but it has been shown to yield more eDNA than precipitation method (Eschmiller et al 2016). Filtration is the most used method with the highest DNA yield. (Xing et al., 2022).

Appropriate storage of eDNA filter samples needs to account for degradation rates in order to best promote eDNA yield during extraction. Common preservation methods include keeping filters in freezing temperatures using ice, liquid nitrogen or freezing samples. Freezing at -20 °C has shown excellent performance, but preservation using buffers and room temperature have been tested, as freezing temperatures are hard to be achieved in field environments. Samples can also be treated with anti-degradation reagents for preservation with ethanol or other lysis buffers. Buffers can be available commercially such as CTAB (CEB-500-02; OPS Diagnostics), RNAlater® (Invitrogen, Sigma-Aldrich, Ambion), and ATL (939011; Qiagen) or pre-made in laboratory like Longmire´s buffer. In general, buffers were shown to preserve samples for up to several months with or without refrigeration (Gorokhova, 2005; Williams et al., 2016; Wu & Minamoto, 2023; Xing et al., 2022).

The next step in eDNA surveys is the extraction of the DNA from intact cells and organelles and purification from substances that can inhibit polymerase chain reaction. Examples of inhibitors vary from cellular components to materials in waters that are co-extracted with DNA, such as humic substances: bile salts in feces, heme group in blood and urea in urine. Those can interfere with PCRs by directly interacting with DNA, by blocking the activity of the polymerase or other PCR mixture components such as Magnesium chloride ($MgCl_2$) (Buckwalter et al., 2014; Eichmiller et al., 2016).

A traditional DNA extraction method is the phenol-chloroform-isoamylol (PCI) method, that has been adapted for use with eDNA samples. Commercial extraction kits are more often recommended to avoid the use of phenol and chloroform, which are harmful substances used during PCI extraction. Commercial kits can be targeted at water samples, soil samples, food, stool or blood and tissue. Example of available kits are Qiagen DNeasy Blood and Tissue Kit, Qiagen DNeasy Power Water Kit, MoBio Power Water DNA extraction kit, MoBio Power Soil DNA extraction kit, MP Biomedicals FastDNA Spin kit for soil, PowerMax Soil kit, Qiagen QIAamp DNA Stool Mini kit, DNeasy Mericon Food Kit.

Molecular tests include a wide range of methods targeting mitochondrial (mt)DNA and nuclear (n)DNA. A wide variety of studies have favored mtDNA due to robustness and to the fact that there is a higher cellular copy number of mtDNA than nDNA (Dalvin et al., 2010; Evans et al., 2017). A short mitochondrial gene for cytochrome c oxidase I (COI 760 bp) has been used as standard for identification of several animal species due to its maternal inheritance and limited recombination, rapid evolution and robustness against degradations (Roe & Sperling, 2007 though targets can range from other regions such as 16S ribosomal (r)RNA or 12S rRNA. Particularly, 16S rRNA has been shown to be superior at detecting sequences to the level of classes and orders than COI (Ahmed et al., 2022; Atkinson et al., 2018).

Quantitative Polymerase chain reactions (PCR) methods for analysis of water samples have been used to detect target DNA fragments using species specific sequences and have successfully detected various taxonomic taxa from family to population level (Strickland & Roberts, 2019).

For quantitative PCR, RT-PCR or qPCR instruments come with an attached fluorescence reader that irradiates samples with ultraviolet light and detects fluorescence levels in every PCR cycle through a camera connected to a system plotting increase in fluorescence real time to accompany the amplification of target DNA. This kind of method is commonly used for detection of eDNA and provide information of concentration of a specific sequence in each sample, requiring an external calibrator or normalization to estimate the concentration of the unknown. The progress can be also followed by the end of each cycle, and the assays for qPCR can be used in field through other platforms than conventional laboratory machinery, such as portable qPCR devices (Hindson et al., 2011).

Droplet digital PCR (ddPCR) is an emulsion PCR method which generates up to 20,000 microdroplets in oil, each droplet being an independent PCR reaction. Positive droplets are defined by high relative fluorescence that results from DNA binding dye detection of double-stranded PCR products. Some reactions will have template while others will not, reactions with the template are a positive endpoint and the number of target DNA molecules will be calculated from those positive droplets using Poisson statistics (Atkinson et al., 2018; Hindson et al., 2011; Te, Chen, & Gin, 2015).

To detect and quantify products, two techniques of fluorescent dyes are commonly used: SYBR Green dye – or EVA Green, that binds to double stranded DNA formed during PCR; and sequence-specific DNA probes, that contain a fluorescent label and add an extra specific component to the assay, as specific hybridization between probe and target sequence is necessary to yield fluorescence (Garibyan & Avashia, 2013). Probe based assays, compared to dye-assays have three sequences checked against target template DNA by the addition to two primers and is known for increasing sensitivity and specificity of the assay.

Hydrolysis probes or TaqMan are common probe assays. Those use minor groove binding (MGB) probes, which provide an additional layer of sensitivity and specificity of the reactions, as three sequences are checked against target DNA instead of two. (Atkinson et al., 2018); (Ahmed et al., 2022; Wilcox et al., 2013).

Biotic factors can impact the capacity of DNA detection and biomass estimation. Seasonality, for example, can provide a broader perspective of presence/absence of taxa when comparing genetic material available in the environment during stages of life-history. As an example, breeding and non-breeding seasons have great impact in genomic concentration which can influence the capacity of detecting and quantifying eDNA. Specially for conservation, it is essential to be cautions regarding when the investigations are conducted as genetic material is more available in different stages of life history for different taxa (Stewart, 2019).

Degradation of DNA is also a common concern when dealing with eDNA samples, potentially causing loss of signal. Decay of eDNA in controlled experiments has been demonstrated to occur on a scale of days to weeks (Sigsgaard et al 2015), other concerns arise such as UV-radiation and inhibitory substances that persist after DNA extraction can bind to the polymerase, interact with DNA and can affect primer extension (Opel et al., 2010). A common practice to address the possibility of PCR reaction inhibition is the use of exogenous control. Spiking samples with positive controls to detect inhibition to observe Cq variation (Bustin et al., 2009) (Andersen et al., 2010).

Studies based on eDNA must also consider of closely related species that can be present in study area. On those cases, sensitivity of the detection method is a crucial step to exclude the possibility of false detection. One example is the use of controls and applying the same technique to different related species (Bustin et al., 2009; Dalvin et al., 2010; Wilcox et al., 2013).

3.4 Aim of this study

The main goal of my thesis is to assess if different eDNA methods can be an effective supplement or alternative to conventional methods in detecting presence and distribution of Atlantic salmon in freshwater systems.

The secondary aims to reach this goal are:

- The evaluation of several eDNA based protocols for detection of Atlantic Salmon in Haugsdalselva during different seasons (winter, spring, summer, and fall). Protocols include different filter types, pore size, eDNA preservation solutions, DNA extraction kits and PCR approaches (quantitative PCR via Real Time PCR and Digital Droplet PCR and probe or dye-based fluorescence).

- Comparison of eDNA analysis with previous reports for Haugsdalselva using conventional methods for detection.

4. Materials and Methods

4.1 Location

Haugsdalselva (Figure 1) is a river in Masfjorden and Modalen municipalities in Vestland county, Norway. It is part of the watershed Haugsdalsvassdraget, where trout and anadromous salmon can reach approximately 4.2 km from the river outlet in Haugsdalsvågen (Masfjorden), up to the migration barrier at Sagfossen (Sægrov et al 2014). It was chosen as a study site as regular assessments are carried out by NORCE – LFI with electrofishing and drift-diving, and spawning areas and salmon presence are well mapped.

Haugsdalsvassdraget has been regulated for hydropower since 1956. This regulation caused a reduced waterflow in Haugsdalselva, but mitigations were implemented during 1990|s that have improved the habitat for salmonids (salmon and trout) spawners and juveniles in the river (Særgrov et al 2014).

The Atlantic salmon population in Haugsdalselva collapsed in the 1980's assumably due to acidification. The water quality in the river gradually improved during the 1990's, and a few juvenile salmon that origin from natural spawners (and not cultivation) was again detected in 2000 for the first time since mid-1980 (Sægrov et al. 2014).

Population of salmon in Haugsdalselva has been assessed annually since 1995 by electrofishing (juveniles) and drift-diving (spawners). These surveys have revealed that the salmon has been re-established, but the population size is still low compared to sea trout (Sægrov et al. 2014; Skoglund et al. 2021).

For my thesis sampling was conducted in five different days in 2020 in the months February, May, August, September, and October, covering the seasons and with two sampling campaigns during spawning time in Autumn. The sampling stations we chose are based on the results from previous assessments that described and detected juvenile and spawning salmon stocks in different areas of this river (Skoglund et al., 2020). Sampling stations are numbered from 1 to 8, the lowest number being closest to the sea (Haugsdalsvågen). Station 7 is upstream the Kjetland bridge, while Station 6 is downstream the bridge. The river flow changed during seasons. Hence station 6 was moved a few meters downstream in summer and autumn to avoid areas with eddy formations and renamed Station 6b.

Station 8 was placed above the migration barrier (upstream Sagfossen) to migrating salmon and sea trout. This part of the river is inhabited by brown trout but no salmon. Hence, this station was chosen

as a negative water collection control for the eDNA method as no salmon eDNA should be expected in this area based on previous assessments. In October, additional sampling stations (St. 5 and St. 5b) were added upstream St. 4 to include same area used in a conventional survey at this time. This is an area that previously has been identified as an important spawning ground for salmon during autumn (see Skoglund et al. 2021).

Sampling stations are shown in Figure 1. Details regarding water sampling and sampling size are presented in Table 1.
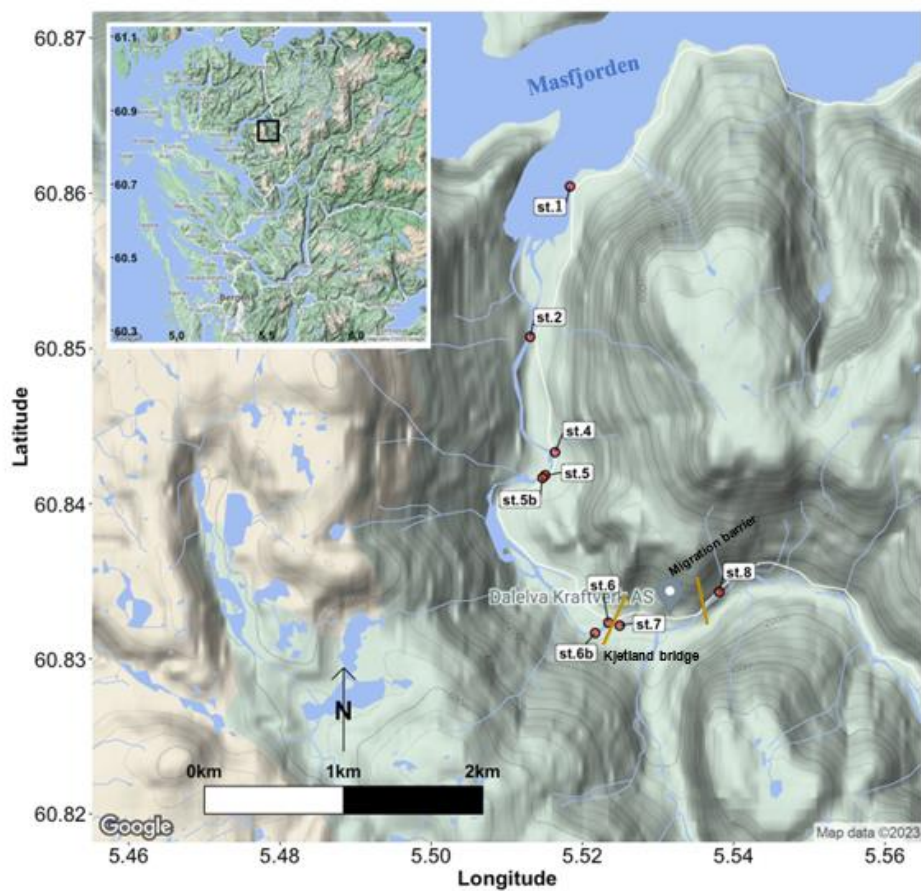

Figure 1 – Map of study location in the river Haugsdalselva, Masfjorden, Modalen municipality in Vestland.

Table 1 −Water sampling in Haugsdalselva 2020. Water volume 5 x 1 litre per station except in August. Filters were 47 mm diameter and types used were Polycarbonate (PC) with pore size 0.45 and Cellulose nitrate (CN) with pore size 0.2, 0.45 or 1.0 µm. In May campaign extra samples were collected to be processed with different extraction kits and preservation solutions. September and October campaigns had extra 3L in two stations for filter pore size comparison. Average water temperature (±SD). *Data provided by Eviny AS. **Temperature measured during sampling. Water samples collected in winter (February) and autumn (September and October) were filtered at NORCE lab in Bergen. Water samples collected in May and August were filtered on-site by the river.

Season	Date	Station	GPS (WGS84) North	GPS (WGS84) East	Average water Temperature °C*	Volume	Extraction and preservation	Filter type and pore size
Winter	February 27th	St.1	60° 51.626	005° 31.101	1,17 ± 0,37*	5 x 1L	PowerWater – PW1	Polycarbonate 0,45 µm
		St.2	60° 51.045	005° 30.785		5 x 1L		Polycarbonate 0,45 µm
		St.4	60° 50.599	005° 30.982		15 x 1L		Polycarbonate 0,45 µm
		St.6	60° 49.940	005° 31.405		5 x 1L		Polycarbonate 0,45 µm
		St.7	60° 49.929	005° 31.496		5 x 1L		Polycarbonate 0,45 µm
		St. 8	60° 50.059	005° 32.286		5 x 1L		Polycarbonate 0,45 µm
Spring	May 5th	St.2	60° 51.045	005° 30.785	5,16 ± 0,98*	20 x 1L	PowerWater | Blood&Tissue	Whatman cellulose nitrate 0,2 µm
		St.4	60° 50.599	005° 30.982		20 x 1L	PW1 – ATL - EtOH	Whatman cellulose nitrate 0,2 µm
		St.8	60° 50.059	005° 32.286		20 x 1L		Whatman cellulose nitrate 0,2 µm
Summer	August 5th	St.4	60° 50.599	005° 30.982	12,92 ± 0,46*	10 x 1L	PowerWater – PW1	Whatman cellulose nitrate 0,2 µm
		St.6b	60° 49.900	005° 31.300		10 x 1L		Whatman cellulose nitrate 0,2 µm
		St.8	60° 50.059	005° 32.286		10 x 1L		Whatman cellulose nitrate 0,2 µm
Autumn	September 30th	St.2	60° 51.045	005° 30.785	11 ± 0,24*	8 x 1L	PowerWater – PW1	Whatman cellulose nitrate 0,45 µm / 1,0 µm
		St. 4	60° 50.599	005° 30.982		5 x 1L		Whatman cellulose nitrate 1,0 µm
		St.6b	60° 49.900	005° 31.300		5 x 1L		Whatman cellulose nitrate 1,0 µm
		St.8	60° 50.059	005° 32.286		5 x 1L		Whatman cellulose nitrate 1,0 µm
Autumn	October 20th	St.2	60° 51.045	005° 30.785	6 °C**	5 x 1L	PowerWater – PW1	Whatman cellulose nitrate 1,0 µm
		St.4	60° 50.599	005° 30.982		8 x 1L		Whatman cellulose nitrate 0,45 µm / 1,0 µm
		St.5	60° 50.510	005° 30.904		5 x 1L		Whatman cellulose nitrate 1,0 µm
		St.5b	60° 50.500	005° 30.883		5 x 1L		Whatman cellulose nitrate 1,0 µm
		St.6b	60° 49.900	005° 31.300		5 x 1L		Whatman cellulose nitrate 1,0 µm
		St.8	60° 50.059	005° 32.286		5 x 1L		Whatman cellulose nitrate 1,0 µm

4.2 Field Sampling and Filtration

River samples were taken from surface water during 5 different sampling trips during the year of 2020. We collected minimum 5 x1 litres of water for each selected site in the river (see Table 1). Samples were collected in 1 L dark bottles to protect them from light exposure. Bottles were transported with ice packs to Bergen for filtration at the NORCE laboratory the same day as sampling or filtered on site.

Samples were collected using disposable nitrile gloves and new gloves were used at each sampling station.

The water samples were filtered using a vacuum filtration station for 47mm diameter filters (KC Denmark) containing a vacuum pump connected to a manifold with six 500 mL acrylic chambers and a tank to collect filtered water (Figure 2). Filtration was conducted in the laboratory for February, October, and September. All filters were placed in 2 ml Cryo tubes (VWR) and filled with 1000 mL PW1 buffer (Qiagen® PowerWater) for preservation prior DNA extraction. An increased sampling size was conducted in May when 20 x 1 litre of water was filtered. Filters from this campaign were placed in 2 ml Cryo tubes (VWR) and preserved with 1000 µl of either PW1 (Qiagen, DNeasy Power Water), ATL lysis buffer ATL (QIAGEN cat. no. 19076) or ethanol for a comparison of preservation effect in DNA yield. The May campaign was also set up to test DNA yield using different DNA extraction methods.

In spring (May) and summer (August) the water samples were filtered on site by the river (set-up shown in Figure 3). After every sample and replicate, the manifold glass columns were rinsed with solution of 10% chlorine (bleach) and MQ water to avoid risk of cross contamination with eDNA between samplings.

When filtration on site, samples were stored on ice in a cooler box during fieldwork and filter samples were stored in a -20°C freezer until DNA extraction.

Different types and pore sizes of filters were used during the water sampling campaign (see Table 1). In February samples were filtered with Polycarbonate 0.45 µm. For the rest of sampling campaigns, we decided for cellulose nitrate filters (CN). These are less expensive and more commonly used for eDNA sampling in fresh water compared to polycarbonate filters (Kumar et al., 2020). Samples collected in May and August were filtered with CN filters pore size 0.2µm and samples collected during autumn (September and October) were filtered using CN filters with increased pore size 0.45 µm or 1 µm, due to challenges with filter clogging.
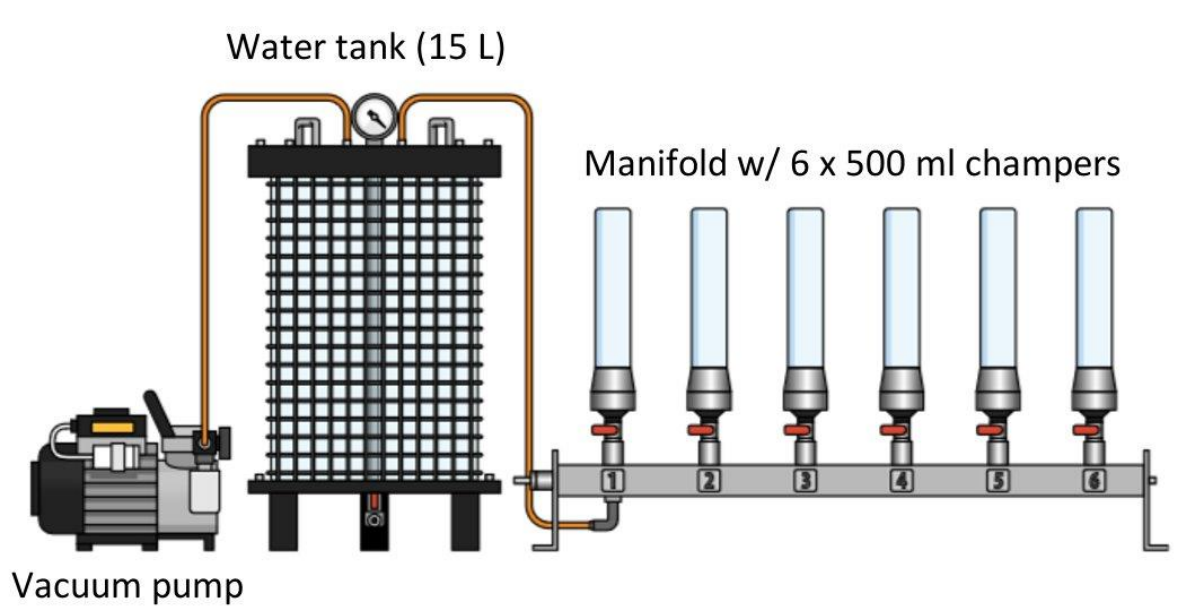
Figure 2 – Vacuum filtration station (KC Denmark). Vacuum pump (125W) connected to a water tank (15L). The water tank collects excess of water during filtration. Six acrylic chambers for filtration using standard 47 mm diameter filter paper.

Figure 3 – Vacuum filtration station set-up in the field. A 12-volt battery (80 Ah) was used as power supply.

4.3 DNA Extraction and quantification of total DNA

5.3.1 DNA Extraction

DNA extraction from filters was conducted in laboratory 1-3 days after water filtration. All samples within one campaign passed by extraction process on the same day. DNeasy Power Water kit (Appendix: Table A) was used in samples from all campaigns. DNA extractions was performed according to the manufacturers protocol (DNeasy® PowerWater® Kit Handbook).

In February, filters from stations 4, 5, 6, 7 and 8 were split into halves; one part was used directly for DNA extraction, and the other half stored in ATL in -80 °C freezer. Filters from station 1, 2 and extra filters (5 L) for station 4 were not split prior to extraction for comparison in DNA yield and inhibitors presence.

5.3.2 Comparative study of different DNA extraction methods

Samples from stations 2, 4 and 8 (4 x 5 L per station) collected during the May campaign for comparison of DNA yield extracted with DNeasy Blood® and Tissue kit (Appendix: Table A) in addition to DNeasy® Blood and Tissue Qiagen and DNeasy® PowerWater (Qiagen) respectively.

Filters from the water collection were preserved in a) PowerWater beads tube filled with 1mL of PW1 for DNA extraction using PowerWater kit, or in 2 mL Eppendorf tubes with either b) ATL or c) Ethanol for DNA extraction using Blood & Tissue kit.

Prior to extraction the filters stored in tubes with ethanol were transferred to new Eppendorf tube with ATL. The tubes with remaining ethanol were then centrifuged and precipitates were transferred to the tube with ATL, required for the first step of DNA extraction with the Blood and Tissue kit.

DNA extractions was performed according to instructions in the respective handbooks for the kits (DNeasy® Blood & Tissue Handbook, DNeasy® PowerWater® Kit Handbook).

Elution of DNA as the final step in DNA extractions was done by adding 100 µL of AE buffer (Blood & Tissue) or 100 µL of EB solution (PowerWater). Two aliquots of 50uL were prepared for each method. One of the samples was storage in -80 °C freezer as backup material, while the other sample was stored in -20 °C freezer until further analyses with PCR methods.

Ultrapure water served as technical extraction controls received the same treatment as samples during DNA extraction for to check for contamination.

5.3.2 DNA Quantification with QuBit

QuBit measures DNA through intensity of fluorescent dyes that bound selectively to target molecules, in this case DNA. Quantification is calculated by comparison based on the Relative Fluorescence Units (RFU) from two standards provided in the assay kit.

Unspecific DNA concentration from filtered water samples was measured using the fluorometer QuBit from Invitrogen, double-stranded DNA (dsDNA) High-Sensitivity (HS) Assay and performed according to the kit instructions from manufacturer's brochure available online. The assay type chosen was the High-sensitivity assay that can detect DNA from 0,1 to 120 ng (0,005-120 ng/μL).

QuBit working solution is prepared using 1:200 of the QuBit DMSO dye stock in the kit´s buffer. Standard solutions are made with 190 μL of the working solution and 10 μL of the QuBit standard reagents. For samples, 198 μL of working solution and 2 μL of undiluted sample. Tubes were mixed by vortex for 2 seconds, centrifuged and incubated at room temperature for 2 minutes. In the fluorometer, standards 1 and 2 were read before running samples. More details of Qubit^{TM} fluoremeter and Qubit assay are given in Appendix (Table A and D).

5.4 PCR

All PCR reaction solutions were prepared in a dedicated PRE-PCR laboratory facility in the University of Bergen not exposed to any PCR products. All equipment and materials were treated for 30 minutes UV light exposition before preparation of PCR mixes. Templates added in each reaction were made of 1:2 diluted samples, ultra-pure water as negative controls and purified DNA extracted from *S. salar* muscle tissue. Sample and controls dilutions were made using TRIS buffer 10 mM (diluted from TRIS 1.0 M, pH 8 from Merck). All samples were performed in triplicates.

Digital droplet PCR (ddPCR) technique consists in partitioning samples into around 20 000 oil-encapsulated droplets and a PCR amplification of each droplet. Droplets can contain one or no copies of the target molecule and during PCR cycles only droplets that contain a molecule will emit fluorescence. Droplets are scored as positive or negative and assigned 1 or 0 values respectively depending on fluorescence intensity above a certain threshold, which can be automatically set by ddPCR analysis software or manually adjusted to differentiate droplets between negative and positive. The quantification is based one the number of droplets at end point and estimated using Poisson distribution (The dMIQE Group et al., 2020).

5.4.1 Quantitative PCR assays

The *Salmo salar L.* assay used in this thesis is a modified version of the assay developed by Atkinson et al 2012 which targeted a region of the COI (Cytochrome Oxidase I) gene from the mitochondrial DNA position 208 5" start position (Appendix: Table B). The amplicon is 74 base pairs in total, including primers. The probe and primer final concentration from the *Salmo salar L.* was modified from 2 μM to 0,2 μM (200 nM) for primers and probe concentration after contact via e-mail with author (S. Atkinson, personal communication, September 23, 2019) and clarification on mistake in publication (published 17. July 2018). The first step of thermocycling conditions (50°C for 2 minutes) was also excluded, also after personal communication with author who confirmed the step to be unnecessary.

Probe-based qPCR: A final reaction volume of 20 μL, being 10 μL SsoAdvanced Universal Probes Supermix, 0.4 μL of each primer and probe, 4 μL sample and ultra-pure water. Cycling conditions were set up to start at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

SYBR Green qPCR: A final reaction volume of 20 μL, being 10 μL SYBR Green Supermix from Bio-Rad, 0.4 μL of each primer, 4μL sample and ultra-pure water. Cycling conditions for SYBR Green were set up to start at 95 °C for 3 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

eDNA samples were run on separate 96-well qPCR plates, each containing positive control and no-template controls. All samples were run in triplicates.

Standard curve was generated using 1:5 serial dilutions from *S. salar* DNA extracted from muscle tissue and quantified with QuBit. Concentration started from 2.5 ng/μL (10ng in 4 μL template) and a positive detection was defined as being in the range of the standard curve.

Probe-based ddPCR: 11 μL of ddPCR Supermix (no dUTP), 0.44 μL of each primer primers and the probe and ultra-pure water. A volume of 5.5 μL as added as template and final volume prepared was 22 μL pipetted to transparent tubes. Thermocycling conditions were set to 95 °C for 1 minute, and 40 cycles of 94 °C for 30 seconds, 56,4 °C for 60 seconds, finishing with 98 °C for 1 minute and infinite hold in 4 °C. Temperature exchanged between steps had a ramp rate of 2 °C per second.

Before thermocycling (ddPCR), a DG8 cartridge (Bio Rad, cat no.:186-4008) was inserted into a holder. A volume of 20 μL ddPCR reaction mix were then pipetted into the sample wells, the middle row of the cartridge. 70 μL of droplet generating oil were pipetted in the bottom wells of the cartridge and placed in a gasket (Bio-Rad, cat. No.: 186-3009) over the cartridge holder. This holder was then placed into the QX200 Droplet generator. After this step, 40 μL of the top row of the cartridge content, with the droplets, were pipetted into a 96-well PCR plate. PCR plate was sealed with Pericable Foil heat seal

(cat no.: 181-4040) using a PX1 PCR Plate sealer. Plate was inserted in thermocycler and after run completion, plate was read with QX200 Droplet Reader.

Genomic DNA from the extreme halophile archaea *Halobacterium salinarum* (LGC Standards GmbH) was used as exogenous control ('spike') for qPCR and ddPCR to examine a) loss of DNA using different kits and b) levels of PCR inhibitors. A specific *H. salinarum* assay (Andersen et al 2010; Appendix, Table B) was used to check for PCR inhibitors by adding a known concentration of *H. salinarum* DNA into the river samples and comparing the difference in qPCR Quantification Cycle (Cq) to a positive control.

Probe-based qPCR: Final volume of reaction was of 20 µL, being 10 µL SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 0.6 µL of Forward Primer (0.3µM), 1.8 µL of Reverse Primer (0.9 µM) and 0.4 µL of Probe (0.2 µM). Thermocycling was set to 98 °C for 2 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds.

SYBR Green qPCR: Final volume of reaction was of 20 µL, being 10 µL SYBR Supermix, 0.6 µL of Forward Primer (0.3 µM) and 1.8 µL of Reverse Primer (0.9 µM). Thermocycling was set to 98 °C for 2 minutes and 40 cycles of 98 °C for 10 seconds and 60 °C for 10 seconds. A melt curve was generated after each run with thermocycling set to 65 °C and gradually increase by 0,3 °C every 3 seconds.

Positive detection is based on PCR results parameters. Quantitative PCR samples below Cq 34.5 for probe-based assay and Cq 32 for SYBR based assays and min 0.25 copies/µL – 5.000 copies/µL and min 3 droplets for ddPCR, according to Bio-Rad manual. Dynamic range was defined using ten 5:1 serial dilution and 8 replicates based on Atlantic salmon muscle tissue, starting from 2.5 ng/µL DNA (total 10ng in a 4uL template and 2ng/µL for ddPCR).

A sample was considered positive if one out of the three technical replicates showed amplification within the C_q value (32cq for SYBR and 34,5 for Probe, salmon assay). Samples with abnormal amplification curves were discarded.

5.4.2 Sensitivity and specificity to the Atlantic salmon assay

Sensitivity and specificity to the Atlantic salmon assay were tested and evaluated for ddPCR and qPCR in a pre-project (Lima et al. 2019). Sensitivity was tested on serial dilutions of DNA extracted from muscle tissue from Atlantic salmon with DNeasy Blood & Tissue kit (Qiagen). Standard curves were made from ten 1:5 serial dilutions with replicates from a 10ng/µL stock (2,5 ng/µL starting concentration for qPCR and 2 ng/µL ddPCR. DNA concentrations were measured with QuBit® dsDNA HS.

Figure 4 - Standard curve for probe-based (Taqman) qPCR assay targeting Atlantic salmon (Atkinson et al. 2018). Serial dilutions (1:5) with eight replicates per plot. Efficiency = 98.4 % for y=22.536 – 3.3604x (R^2 = 0.99).
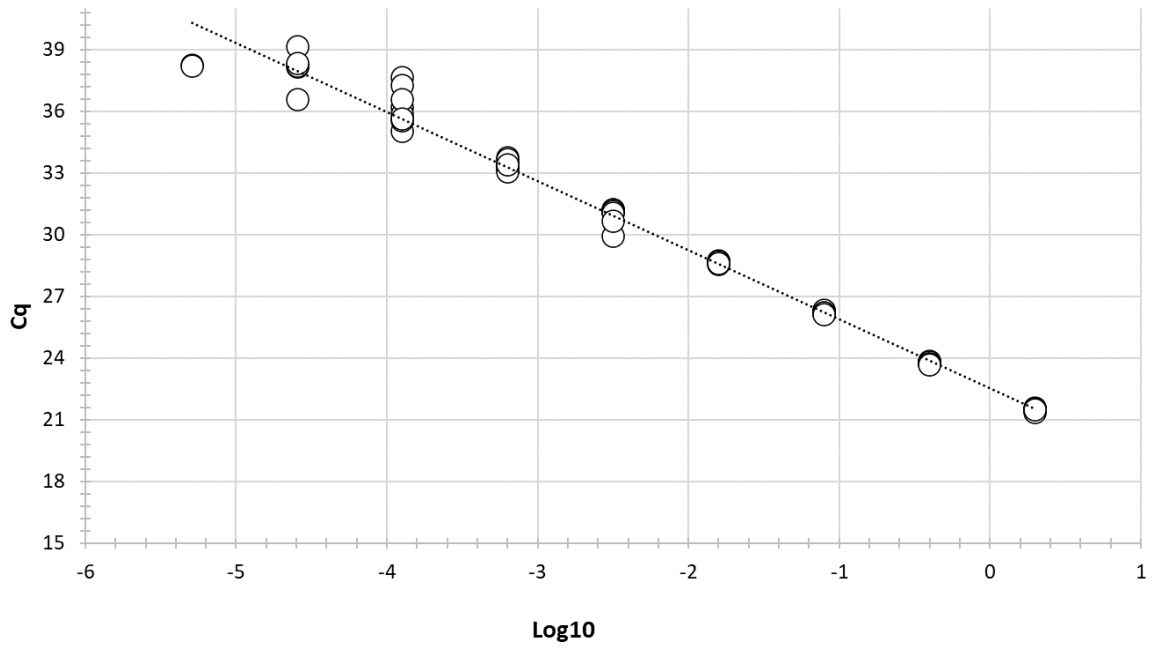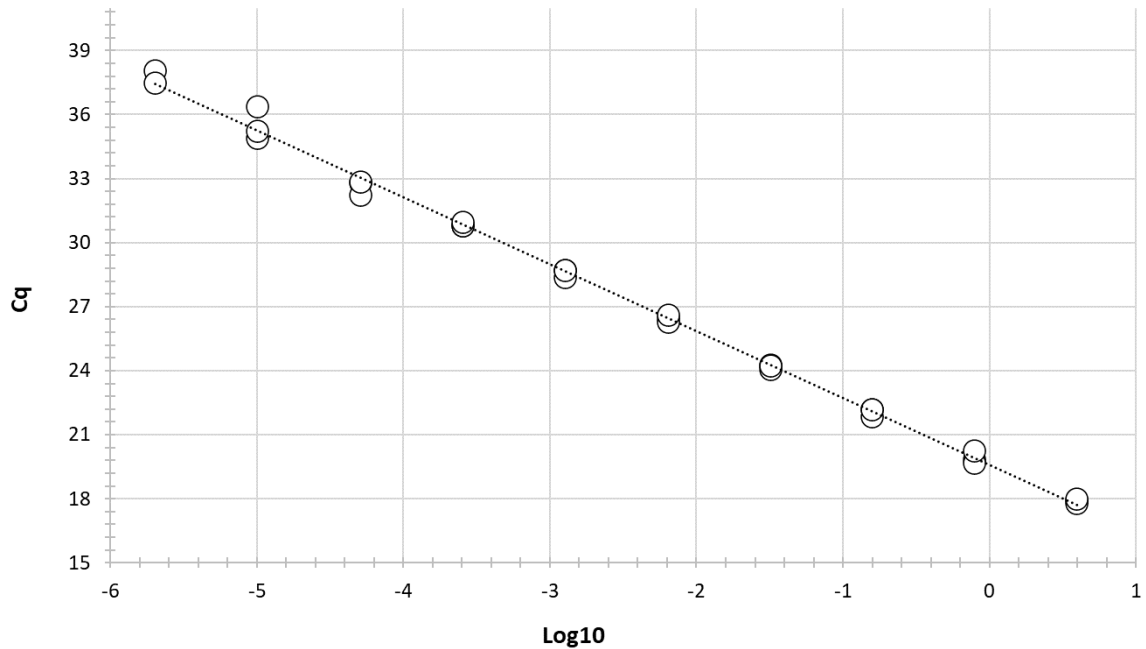

Figure 5 - Standard curve for SYBR green based qPCR assay targeting Atlantic salmon (Atkinson et al. 2018). Serial dilutions (1:5) with three replicates per plot. Efficiency = 108.3 % for y=19.568 – 3.1382x (R^2 = 0.99). Data obtained from Lima et al. (2019).

Efficiency and sensitivity to the assay is determined by the standard curves. Limit of detection (LOD) is defined as highest Cq values where at least 95% of the replicates are positive (Forootan et al., 2017). LOD in the present study is defined as highest Cq values where all triplicates are positive (mean value). Hence, LOD for the Atlantic salmon assay is set to Cq 36 (8 of 8 replicates) and 37 (3 of 3 replicates) for probe-based qPCR and SYBR green qPCR respectively (see Figures 4 and 5).

Specificity to the assay was tested against 16 fish species from 6 different taxonomic orders including the salmonids Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), and pink salmon (*Oncorhynchus gorbuscha*). Atlantic salmon assay sensitivity and specificity followed this previous work defined a detection limit of Cq 34.5 for probe based (Taqman) qPCR and Cq 32.0 for SYBR green qPCR . That is, values above these limits will increase risk of false positive. Hence, a cut-off for the assay is set to be 34.5 and 32.0 for Cq values obtained with probe based and SYBR Green qPCR respectively.
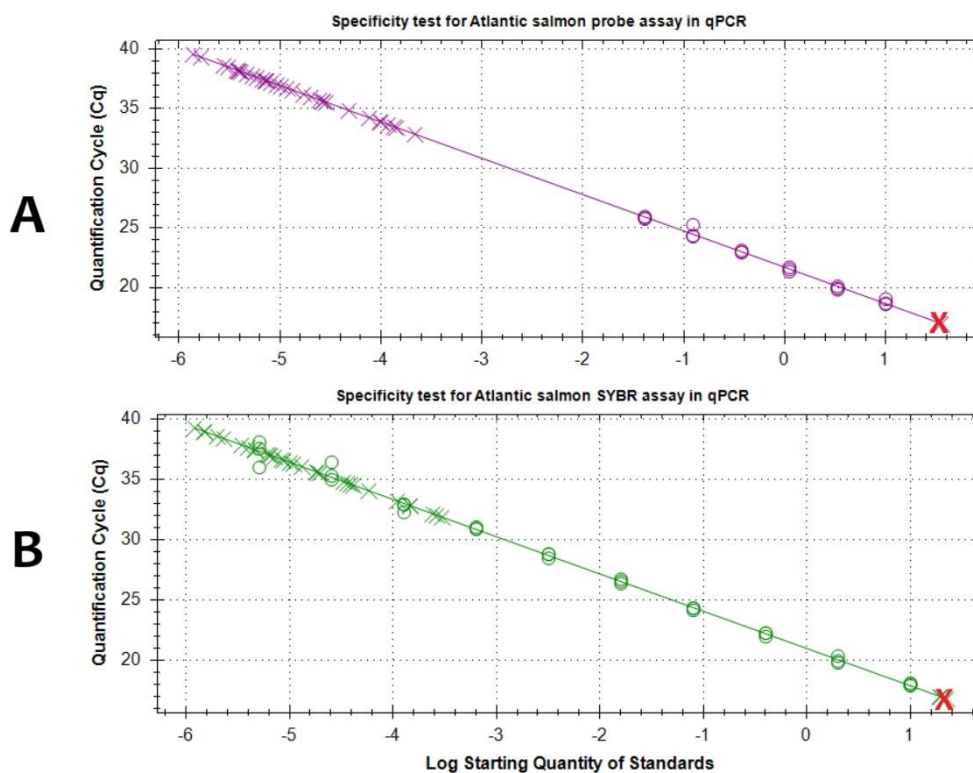
Figure 6 –Specificity test for Taqman probe-based (A) and SYBR-based (B) qPCR using assay targeting Atlantic salmon (Atkinson et al.(2018)). Standard curve for 5-fold serial dilutions of Atlantic salmon DNA (circles) with a start concentration of 0.5 ng/µL. Undiluted DNA (concentration 2.5 ng/ µL) from other fish species (including salmonids). Detections of undiluted fish DNA marked with X in the figure (red X is Atlantic salmon; green or purple X are other fish species). DNA extracted with DNeasy Blood & Tissue (Qiagen). Data obtained from Lima et al. (2019).

For ddPCR, mean concentrations defined the range of detection (Figure 7). Sample concentration started from 2 ng/uL stock solution as ddPCR template and ranged from 1:5 to 1:1 953 125. Range of detection fell on average between 3644 copies/uL (1:5) to 0.157 copies/uL at dilution 1:78 125., when < 3 droplets are observed. According to user manual from Bio-Rad, LOD for ddPCR is 0.25 copies per µl and minimum 3 positive droplets. Cut-off for ddPCR results was based in previous specificity tests, with unspecific amplification at 0.36 copies/uL and 4 droplets.

Both for ddPCR and probe-based qPCR dynamic range was until a DNA concentration of dilution 1:78125.

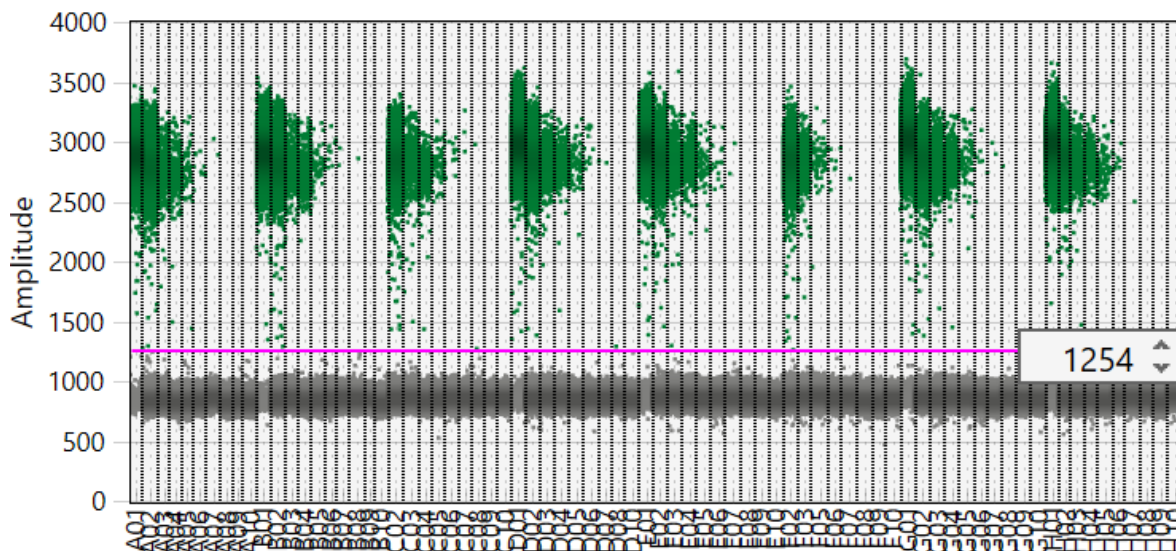Figure 7 - Sensitivity test of Atlantic salmon assay in ddPCR runs. Amplitude plot from QX Manager and BioRad Maestro dilution series to determine the range of detection for the assay targeting Atlantic salmon with use of ddPCR. Starting from 2 ng DNA (undiluted positive control with 10ng exceeds ddPCR range of detection). Specificity test set minimum concentration at 0.36 copies/uL. Limit of detection 0,157 copies/uL.

5.4.3 Assay testing in water samples

To test the assay with eDNA to detect Atlantic salmon, probe assay via qPCR and ddPCR, eDNA samples that were collected from fish tanks in the ILab at the University of Bergen.

Water samples originated from two tanks: one with cod, 78 individuals weighting approximately 450g in 7500 liters of water (exchange rate of 6500 liter/hour), water originated from 100m depth from Nordnes, Bergen, treated with UV light before entering the tank. Temperature at 9.3 ^{o}C in the sampling day.

Salmon was filtered from a 2500-liter tank with a full shift of water per hour. Water was fresh from Masfjorden and contained 400 fish weighing approximately 110g and the temperature was 12°C.

Concentration of samples were of 2.31ng/µL from cod tank and 1.75ng/µL in salmon tank. Some of the QuBit measurements from Haugsdalselva have the same or around the same concentration – from May to October. From both samples, 5,5 µL was used as template for ddPCR and 4 µL to qPCR.

5.6. Controls

5.6.1 Negative and contamination controls

As biological negative control, we collected water samples from Station 8, a portion in the river with confirmed absence of Salmon according to previous studies and reports from Rådgivende Biologer AS and NORCE LFI (Skoglund et al. 2019, Skoglund et al. 2020; Sægrov et al. 2014).

Five litres of distilled water were brought to every campaign and received the same treatment as samples to serve as negative technical sampling control.

Contamination controls were included in several steps of this study to determine and mitigate the influence of contamination in samples or tests. The controls consisted of Milli-Q Ultra-pure water that received the same treatment as samples. We carried 1 L of ultra-pure water in five different bottles from the laboratory to the sampling field to be treated identically to the sampled bottles during transport, filtration, DNA extraction and PCR runs.

Ultra-pure water used for PCR mixes preparations, extraction controls made of MiliQ-water and TRIS buffer used in dilutions also underwent PCR tests. Controls added in all steps in the workflow were used to identify any possible background contamination in preparation, transportation, filtering, dilutions, and PCR runs (Figure 4).
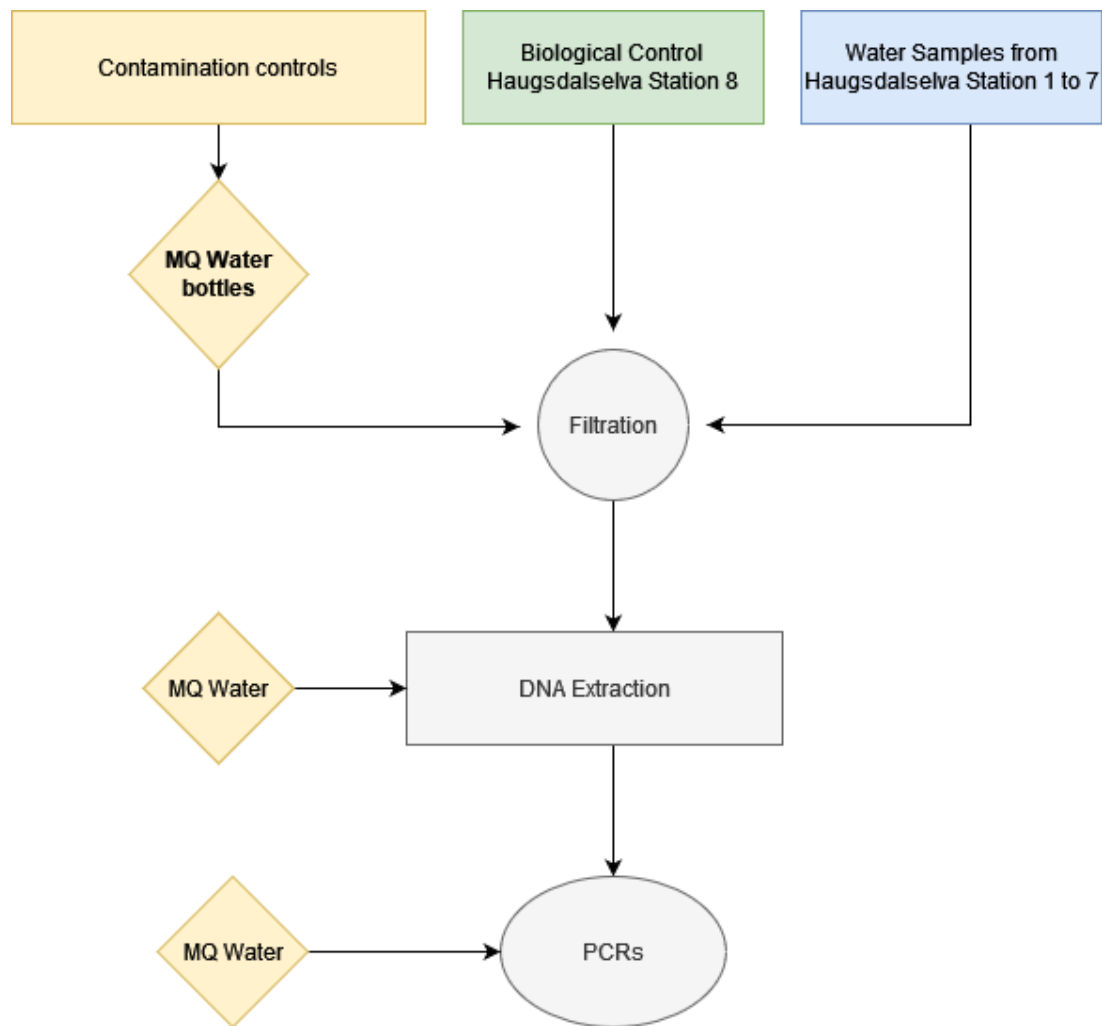
Figure 8 – Diagram of the biological and technical controls included in every step of the workflow from sampling to PCR analysis. Controls underwent the same treatment as river samples during extraction, filtration and PCR runs. Ultra-pure water used in PCR mixes and TRIS buffer used in sample dilution were also included in PCR runs to check for contamination.

5.6.2 Positive controls

Positive controls were included in all runs in order to detect reagent mix errors. DNA from *Salmo salar L.* muscle tissue was extracted with Qiagen DNeasy Blood and Tissue kit used as positive control for all PCR runs using *S. salar L.* assay. Before extraction, muscle tissue was treated with TissueRuptor II system from Qiagen (Figure 5). TissueRuptor is a rotor-stator homogenizer for disruption and homogenization of biological samples through turbulence and mechanical shearing operating in a speed range of 5 000 to 35000 rpm.

I pippeted 20 µL of Proteinase K in a 5 mL Eppendorf tube containing the muscle tissue in ATL buffer and placed in a heating block at 56 °C overnight. Tissue disruption was performed according to manufacturer's user manual using a disposable plastic probe is installed onto the motor unit.
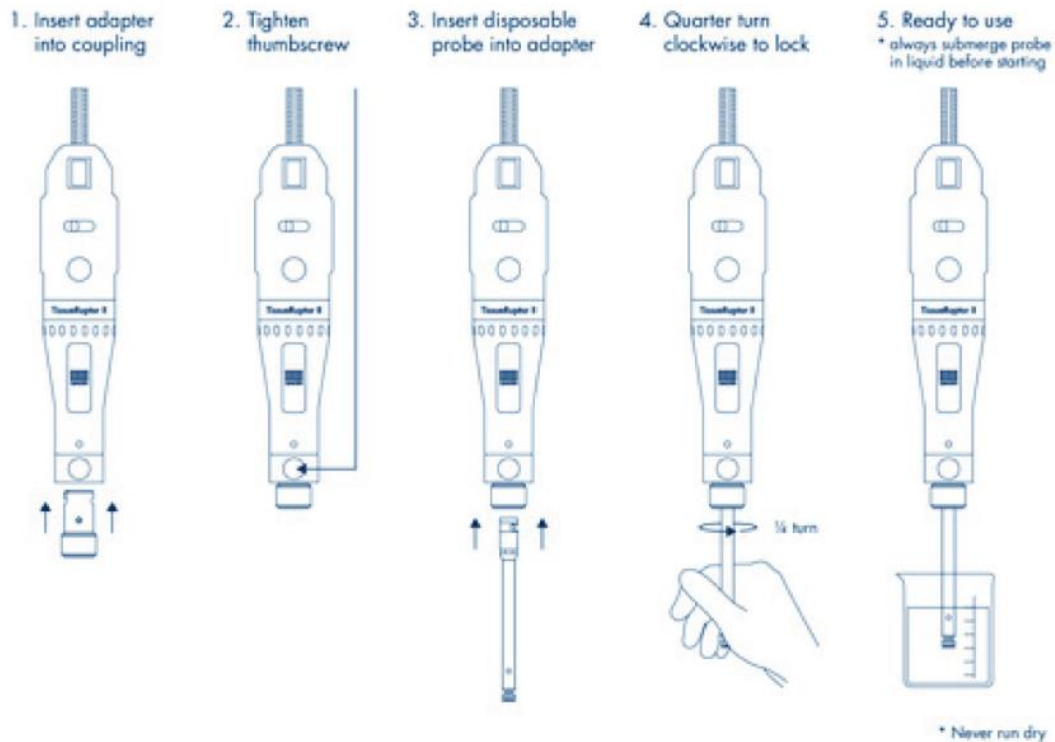
Figure 9. TissueRuptor II probe installation using adapter. Image from Qiagen User Manual 03/2022. After use of the TissueRuptor, I pippeted 180 μL of the processed muscle into twenty 2mL Eppendorf tubes to run the DNA extraction. MiliQ water was used as an extraction blank and underwent the same treatment as muscle samples.

I made 20 aliquots to be extracted and an extraction blank to check for contamination. After DNA extraction, each sample was divided into 2 aliquots of 50 μL.

Purified *S. salar L*. DNA was measured with QuBit and normalized to 2,5 ng/μL for use with qPCR and 2ng/μL for ddPCR to obtain 10ng DNA in final PCR solutions when undiluted.

I made a serial dilution of 1:5 used as positive controls dilutions 1:25 or 1:125, to avoid saturation. Samples were stored in -80 ºC freezer until used to make positive control dilutions, that were stored in -20 ºC freezer.

5.7 Workflow

A summary of experimental design and steps from sample collection to PCR analysis is presented in Figure 6 and shortly described in the following: River sampling was conducted in Haugsdalselva 2020, during Winter, Spring, Summer and Autumn. Two sample campaigns happened in Autumn, in September and October as it is spawning time for Atlantic Salmon and has been show from previous reports as the period adult fish return to the river to reproduce (Skoglund et al. 2019; Skoglund et al. 2020). Water was filtered in site or in lab using a vacuum pump connected to a tank and manifold with 6 champers. Filters used were made of Polycarbonate with a 0.45 μm pore size and cellulose nitrate with 0.2 μm, 0.45 μm or 1 μm.

DNeasy PowerWater (Qiagen) extraction kit was used on filters from all stations and extra samples were collected in May to be tested with DNeasy Blood and Tissue (Qiagen) conserved in ATL or Ethanol for comparison. Samples were then tested with QuBit for total DNA quantification. Final analysis was done using two different PCR quantification instruments (ddPCR, qPCR), based on two different fluorescence-based assays (SYBR/EvaGreen and Probe) and with two different targets:

Salmo salar L. mtCOI gene for salmon detection in Haugsdalselva and *H. salinarum* (spike) to measure inhibition.
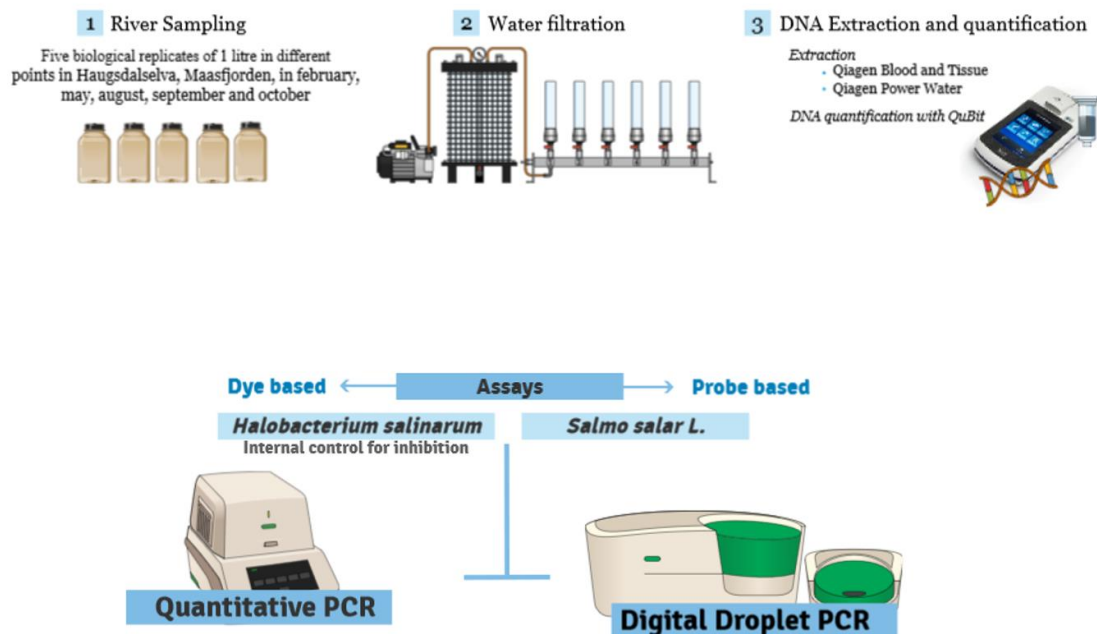

Figure 10 – A summary of the workflow from water sampling to PCR analyses.

5.8 Data analysis

Statistical analysis of DNA concentrations, PCR output comparisons were performed using R version 4.2.2 (developed by Bell Laboratories by John Chambers and colleagues) an open-source software available as Free Software under the terms of Free Software Foundations GNU General Public License using RStudio version 2022.12.0 + 353 "Elsbeth Geranium" integrated development environment (IDE) (Posit Software, PCB).

Two-way ANOVA test was used to detect significant difference between results of DNA concentrations measured with QuBit for different stations, seasons, and extraction kits. For detailed significant difference between groups comparison, I used Tukey HSD. Significant results were based on p value when < 0.05. For two-group comparisons, I used Welch's t-test.

Results from ddPCR tests were analysed using QX Manager 1.1 Series for Bio-Rad's Droplet Digital PCR and for qPCR results, CFX Manager 3.1.

Positive detection is based on PCR results parameters. Quantitative PCR samples below Cq 34,5 for probe-based assay and Cq 32 for SYBR based assays and min 0.25 copies/µL – 5.000 copies/µL for ddPCR and minimum 3 droplets, according to Bio-Rad manual and dynamic range tests based on Atlantic Salmon muscle tissue and 5:1 dilutions starting from 10 ng of the target DNA to 5.12e-6 ng for ddPCR and qPCR.

I used a similar approach to Jacobsen (2023) for Limit of Detection (LOD) and Limit of Quantification (LOQ). One technical replicate qualifies that replicate to be considered positive. In order to successfully quantify qPCR product, all replicates need to be positive (LOQ).

ddPCR thresholds were chosen every plate, manually, considering Kokkoris et al (2021) mentions on the challenges of setting threshold values for environmental samples in ddPCR. Besides the negative droplets population from my NTCs, my threshold setting every run took into consideration my negative biological control.

6. Results

A total of 156 L of water samples was collected, filtered, and tested for presence of Atlantic salmon DNA in Haugsdalselva during 2020. This study shows substantial variations in Atlantic salmon eDNA concentrations among samples collected from different sites in the river at different seasons.

In addition to spatial and temporal variations, I also identified methodological factors that have significant impact on eDNA yield and detection accuracy in water samples from the river. These factors include methods regarding filtration, filter preservation, DNA extractions and PCR (qPCR, ddPCR).

6.1 Unspecific DNA concentration variability during seasons in Haugsdalselva river

Water samples from different stations was collected and filtered during winter, spring, summer, and autumn (Table 1). In all campaigns, filters were preserved in PW1 and DNA extracted using Qiagen PowerWater. Five replicates of 1 litre water from each sampling station was filtered, and during August campaign filtering time increased considerable from 15 minutes per station in February and May to an average of 30 to 45 minutes in August, with one filter in station 6 taking 60 minutes. This increased filtering time was caused by filter clogging. Figure 7 shows example of CN filters from May (no clogging) and August (clogging).

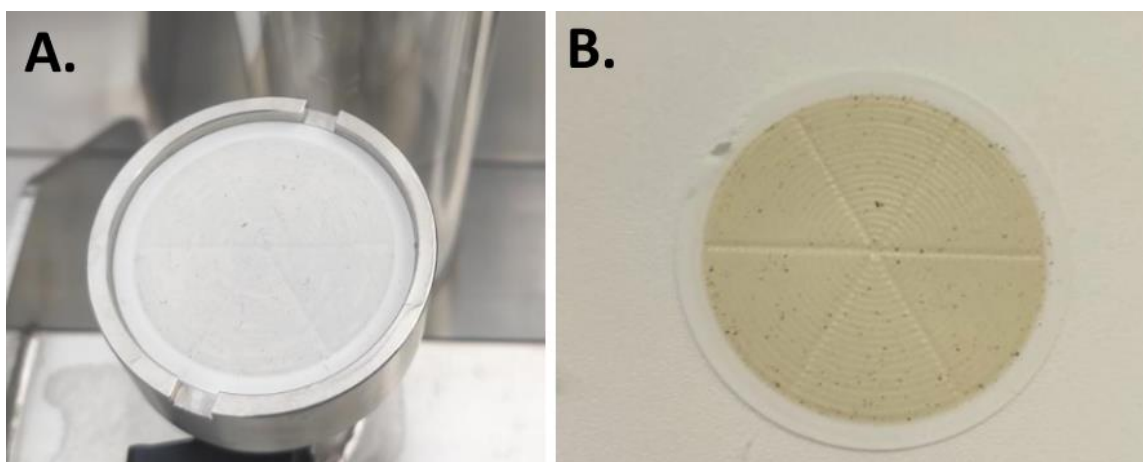Figure 11 – Comparison of CN filters used in May (A) and in August (B) with 0.2 μM pore size. In May (A), filter sits on manifold and has a light coloration from particles. Filter in picture B is from an August sample and it is much darker pointing out the higher biomass and particles quantity present in summer. This is also reflected in filtration times in August, that took the longest among the campaigns.

Measurements of unspecific eDNA concentrations in the water samples revealed both temporal and spatial variations (Figure 12). There was a significant effect of seasonality in eDNA yield in Haugsdalselva by month (two-way ANOVA; ($F_{(4,99)}$ = 44.15, $p<0.001$). In overall, there was significant lower DNA concentrations in water samples collected in February compared to water samples collected in August and October($p<0.05$).

There was also significant difference in DNA concentrations in water collected from different sampling stations ($F_{(8,99)}$ = 6.65, $p < .001$), station 6 yielded the highest concentration of DNA when compared with all stations from February and stations 4 and 8 in August (<0.001).
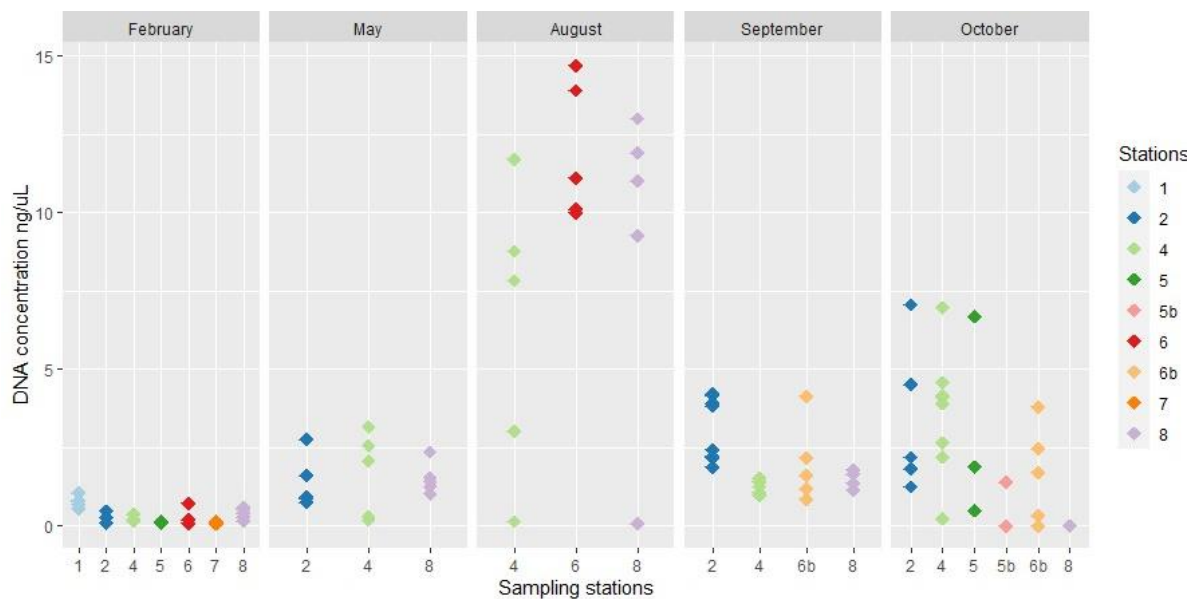
Figure 12 – Water samples. Unspecific eDNA concentration variability during seasons at different sites (Stations) in Haugsdalselva. Replicates (5 x 1 litre) of water filtered. Extracted concentration of DNA given as ng per µL water. DNA extraction with Power Water (QIAGEN). DNA concentration measured with QuBit assay.

6.2 Comparison of DNA extraction methods

From samples collected in May for stations 2, 4 and 8 to check DNA yield using different extraction and conservation methods, I found that DNeasy PowerWater has shown a higher DNA yield compared to the DNeasy Blood and Tissue extraction kit.

Extra filtered water samples were collected at station 2, 4 and 8 in May for comparisons of DNA yield achieved with two DNA extraction methods, namely DNeasy PowerWater and DNeasy Blood & Tissue Filters for PowerWater kit were conserved in PW1 buffer provided as part of the kit, while filters for Blood & Tissue were conserved in ATL lysis buffer provided with the kit. In addition, extra filter samples were conserved in ethanol prior DNA extraction using the Blood & Tissue kit.

Results show a clear distinction in unspecific DNA yield between the different DNA extraction methods. Measured DNA concentration from samples conserved in ATL or ethanol (for DNA extraction using Blood & Tissue kit) show DNA yields lower than 1 ng/µL for all replicates, while the DNA yield in samples conserved in PW1 (for DNA extraction using PowerWater kit) are ranging up to 3.16 ng/µL. In overall, there were significant differences in eDNA yield when different extraction methods for samples from all stations were compared (two-way ANOVA; p<0,001, 95% CI). This effect only corresponds to extractions methods, showing a significant higher eDNA yield in samples where DNA were extracted using the DNeasy Power Water kit compared to the DNeasy Blood & Tissue kit (Table 2 and 3).

There were no significant differences in DNA yield from samples conserved in ATL lysis buffer or ethanol prior to DNA extraction with use of Blood & Tissue kit. No effect of different stations (St. 2, St. 4 and St. 8) or interaction between stations and conservation methods were observed (Table 3).

Differences in measured DNA concentrations from samples collected at different stations and different conservation methods are shown in Figure 13.
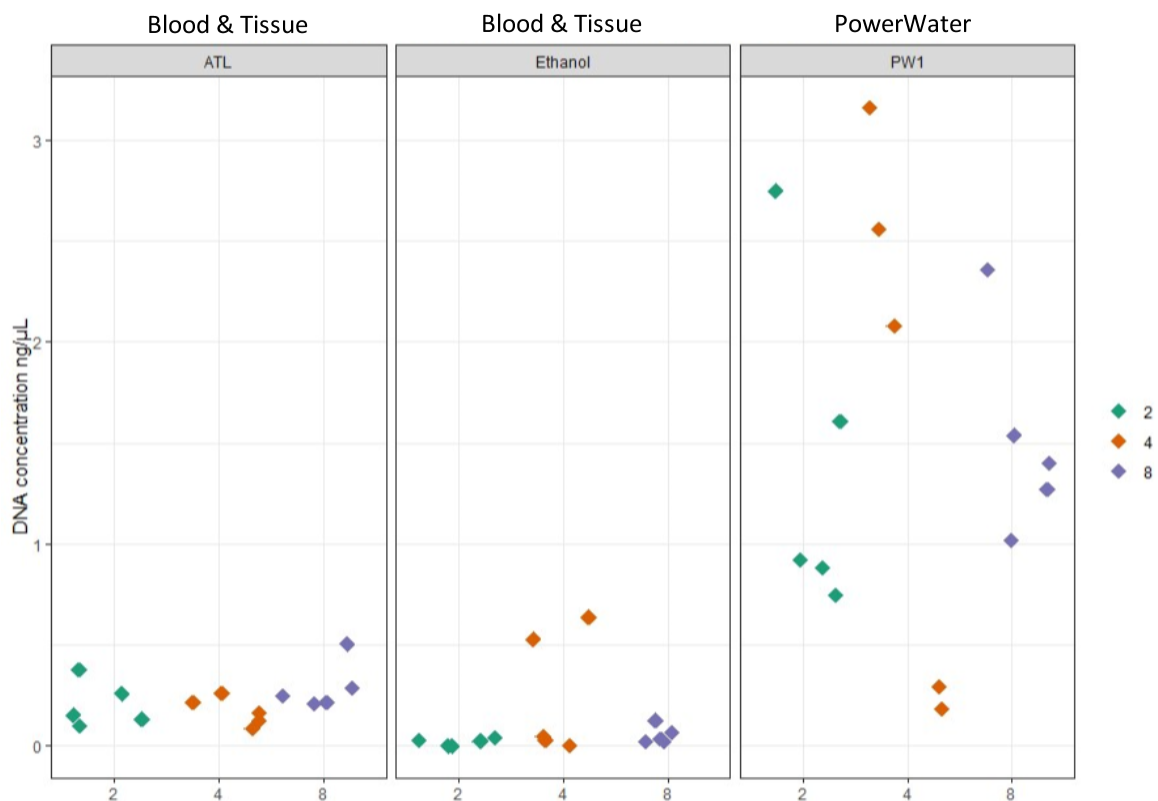
Figure 13 – Comparison of unspecific DNA yield from different conservation and extraction methods. Water samples collected and filtered from different sites (sampling stations numbered as 2, 4, and 8) in Haugsdalselva, May 2020. DNA from the filters were extracted using Qiagen DNeasy PowerWater (filters conserved in PW1 prior DNA extraction) and Qiagen DNeasy Blood & Tissue extraction kits (filters conserved in ATL or ethanol prior DNA extraction). DNA concentration (ng/uL) measured with Qubit (dsDNA HS assay). Plotted points represent biological replicates (filter samples; 3 x 5 replicates per station).

Table 2 – DNA yield. Preservation of filtered water samples from different sites (Stations) in Haugsdalselva. A total of 15 L samples was filtered and preserved in ATL lysis buffer or ethanol for DNA extraction using DNeasy®Blood & Tissue kit and in PW1 buffer for DNA extraction using DNeasy® Power Water kit. DNA concentrations measured with Qubit fluorometer are given as ng per µL and are given as mean ± standard deviation. Coefficient of variation (CV) given in percent.

Station	Conservation	Filter samples (n)	DNA Conc (ng/µL). Mean ± SD	Coeficient of variation (%)
	ATL	5	0.206 ± 0.11	55.2
2	Ethanol	5	0.0188 ± 0.0182	96.7
	PW1	5	1.38 ± 0.835	60.4
	ATL	5	0.171 ± 0.0696	40.7
4	Ethanol	5	0.250 ± 0.309	123
	PW1	5	1.66 ± 1.35	81.4
	ATL	5	0.293 ± 0.123	41.8
8	Ethanol	5	0.0550 ± 0.0452	82.2
	PW1	5	1.52 ± 0.508	33.5

Table 3 – Conservation and extraction method comparison for eDNA yield. A two-way ANOVA shows that only conservation/extraction methods influence eDNA yield. Differences between stations and interaction of station and conservation had no effect (p>0.05) in the final measure. Last column shows p-value for the impact of conservation (p<0.001). Statistically significant for p<0.05 *.

	Degrees of freedom	Sum of Squares	Mean Squares	F value	p-value
Conservation	2	18.41	9.20	28.53	0.0000*
Station	2	0.19	0.09	0.29	0.7521
Conservation:station	4	0.20	0.05	0.15	0.9604
Residuals	36	11.61	0.32		

6.3 Assay test in water samples

Tests with water samples from fish tanks with known high concentration of eDNA resulted in a dramatic difference between salmon and cod, proving the assay to be specific within the range previously defined for both species (Table 4). Those very low concentrations of non-specific eDNA show the importance of setting cut-off limits, as in literature many studies don't cite exactly the protocols used for assay specificity(Atkinson et al., 2018; Fossøy et al., 2020; Jacobsen et al., 2023; Sigsgaard et al., 2015) or still use extremely high Cq for qPCR (Sigsgaard et al., 2015) non-recommended due to non-specific amplification and decrease of efficiency (Bustin et al., 2009). For ddPCR, the manufacturer manual suggests <0.25 copies/uL and three droplets in one or merged wells to call a sample positive. Many studies use this threshold as successful detection (Fossøy et al., 2020; Hansen et al., 2022).

Table 4 – eDNA water samples from fish tanks with cod and salmon were measured for DNA concentration with QuBit dsDNA HS assay and tested with probe assay in qPCR and ddPCR. Concentration is unspecific and refers to total DNA in the sample. Results for qPCR show the average Cq between replicates, cut-off previously set as 34,5. ddPCR results are divided between Copies per microliter and positive droplets, cut off 0.36copies/uL or 3 positive droplets.

Water samples ILAB	DNA Concentration	qPCR	ddPCR	
		Average Cq	Copies/uL	Positive Droplets
Salmon	1,75 ng/uL	$24.76 \pm 0{,}17$	534.78	4544
Cod	2,31 ng/uL	37.01 ± 0.35	0.09	1

6.4 Detection of inhibitors

Halobacterium salinarum assay was used as exogenous control (spike) to measure loss of DNA during DNA extraction (DNeasy PowerWater) and to control for PCR inhibitors in water samples.

When comparing inhibition between seasons in Haugsdalselva, the month of February shows lowest inhibition using qPCR probe assay, with the average Cq closer to the positive control, 24. Higher Cq values and high data spread within campaign signal presence of PCR inhibitors. Spatial and temporal variations in levels of PCR inhibition in water samples from Haugsdalselva are shown in Figure 10.
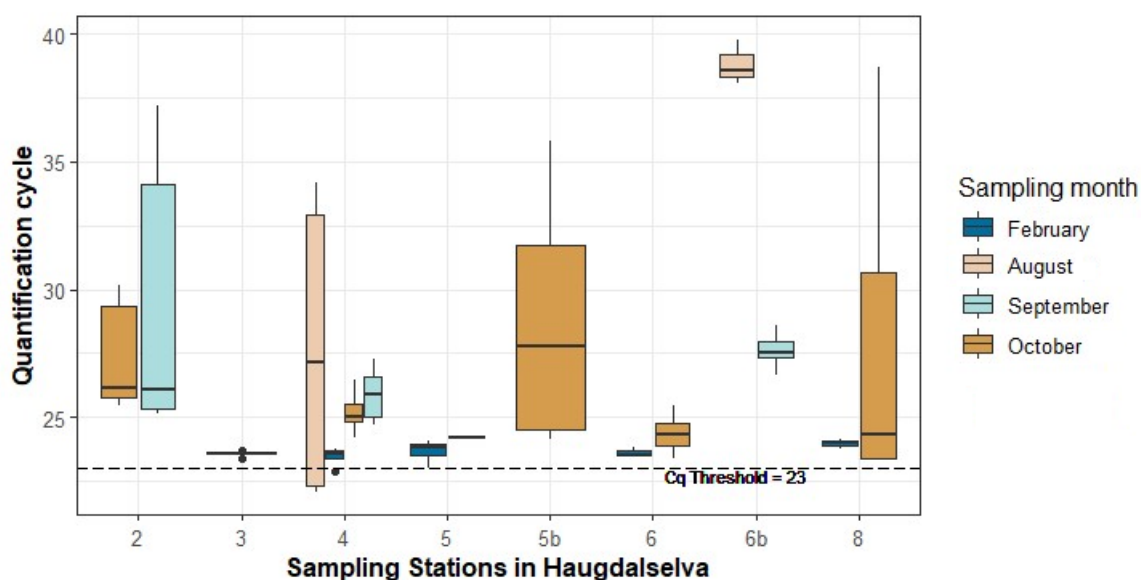
Figure 14 – PCR inhibition test. Quantification cycles in qPCR assay for spike (*H. salinarum,* exogenous control) show February as the month with the least inhibition, with its values slightly above the threshold. followed October, September, and August.

Samples from May were spiked with 2uL of 0,025 ng/uL of *H. salinarum* and analyzed with qPCR to compare inhibition between samples treated with DNeasy Power Water and DNeasy Blood and Tissue. *H. salinarum* assay probe assay. Only samples treated with Power Water showed amplification when analyzed undiluted. Distance from the y intercept line (Control Cq = 17.4) corresponds to presence of inhibitors in a sample (see figure 15).

Figure 15 –Sample dilution and extraction kit influence on qPCR quantification cycle for assay targeting *Halobacterium salinarum* in spiked samples. Water samples were collected in May at station 4 in Haugsdalselva. PCR inhibition test was performed with qPCR probe assay targeting *H. salinarum*, and qPCR runs on undiluted DNA sample and on DNA samples diluted 1:10 and 1:100. Dashed li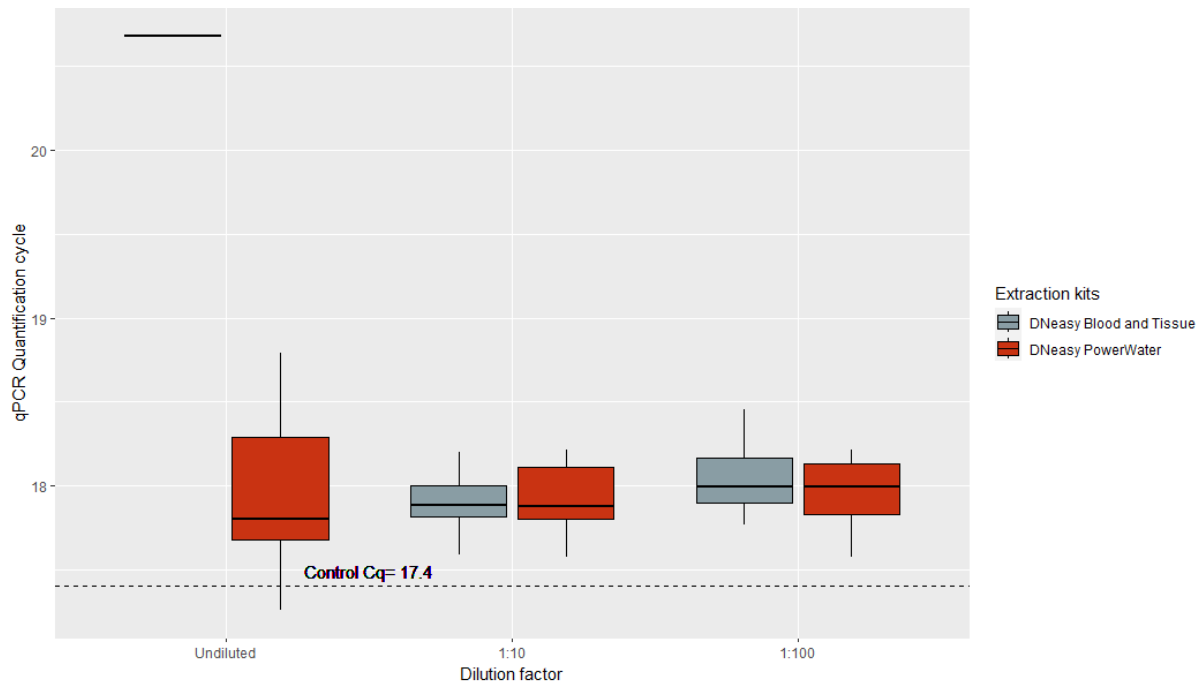ne represents the quantification cycle (Cq) of positive control sample of *H. salinarum*. Water samples from Haugsdalselva and positive control (ultra-pure Milli-Q® water) were added with the same concentration of *H. salinarum* DNA.

6.5 Detection of Atlantic salmon DNA in Haugsdalselva

Atlantic salmon was detected in all seasons in Haugsdalselva in all methods at the same or different time points and biological replicates (Table 5).

Station 4 has the highest proportion of successful detections among replicates in all months. The second station that detected salmon successfully in all seasons was Station 2. There were significant higher proportions of detections with ddPCR than qPCR in samples from station 4 collected in august and in samples from station 2 collected in October (FET; p<0.001). Summer and early autumn were the seasons with the least number of detections in all methods.

Table 5 – Overview Salmon detection in Haugsdalselva in 2020 using probe-based assay for both qPCR and ddPCR. Table is divided into seasons and stations. Results are given in station positive replicates (biological replicates), average Cq value ± Standard deviation for qPCR and copies/uL for ddPCR, and proportion of total positive PCRs among biological and technical replicates. Cq value 34.5 set as cut-off for the specificity to qPCR. Cut-off for ddPCR is set to 0.36 copies per µl and minimum 4 droplets. Differences in numbers of positive detections for qPCr and ddPCR tested with Fisher Exact test (FET). Differences statistically significant for p<0.05*.

Season	Date	Station	qPCR			ddPCR			FET
			Station positive replicates	Average Cq value ± SD	Positive detections	Station positive replicates	Average copies/ul ± SD	Positive detections	
Winter	27th February	1	-	-	-	-	-	-	
		2	1/5	34.36	1/15	1/5	0.38	1/15	1.000
		4	5/10	34 ± 0.39	10/30	5/10	0.57 ± 0.18	9/30	1.000
		5	-	-	-	-	-	-	
		6	-	-	-	-	-	-	
		7	-	-	-	-	-	-	
Spring	5th May	2	3/5	33.41 ± 030	7/15	3/5	1.14 ± 0.56	9/15	0.715
		4	3/5	33.48 ± 0.21	9/15	3/5	1.16 ± 0.36	9/15	1.000
Summer	5th August	4	1/5	34.24	1/15	4/5	0.68 ± 0.21	11/15	0.0005*
		6b	0/5	-	0/15	1/5	0.39	1/15	1.000
Autumn	30th September	2	1/5	33.46 ± 0.9	3/15	2/8	0.44 ± 0.11	2/24	0.354
		4	1/5	33.27 ± 0.9	3/15	2/5	0.45 ± 0.61	3/15	1.000
		6b	1/5	34.25	1/15	0/5	-	0/15	1
Autumn	20th October	2	3/5	33.03 ± 0.43	8/15	5/5	2.18 ± 1.39	15/15	0.006*
		4	5/5	32.72 ± 073	15/15	5/5	0.79 ± 0.34	15/15	1.000
		5	2/3	31.85 ± 0.88	6/9	2/3	1.06 ± 0.88	4/9	0.637
		5b	1/3	34.5	1/9	1/3	0.64 ± 0.1	3/9	0.577
		6b	0/5	-	0/15	0/5	0.13 ± 0.06	0/15	1.000

6.5.1 Influence of sample dilution for detection of Atlantic salmon

Samples from October, stations 4, 5 and 6b, were diluted before analyses and compared with undiluted ones, to access effects of inhibition in samples and the impact on overall detection of Atlantic salmon by qPCR using the probe assay.

Diluting samples has not impacted negatively the detection and in comparison retrieved more positive detections (in qPCR) than undiluted samples.

Table 6 – Haugsdalselva 2020. Triplicate samples from stations 4, 5 and 6b tested with probe-based qPCR assay (Atkinson et al 2018) and treated to 2-fold dilution and samples undiluted.

Month	Site	Cq	Average Cq (n=3 technical replicates)	Positive PCR
October Diluted 1:2	4	34.07 | 33.15 | 33.28	33.53 ± 0.49	3/3
	5	32.58 | 32.73 | 33.67	32.82 ± 0.44	3/3
	6b	36.62 | 34.52 | NA	35.57 ± 1.48	1/3
October Undiluted	4	NA	NA	0/3
	5	31.22 | 31.97 | 30.69	31.39 ± 0.64	2/3
	6b	36.05 | 36.78 | 36.86	36.55 ± 0.44	0/3

6.5.2 Comparison of SYBR and Probe qPCR assay for salmon

In the month of October, the SYBR Green based qPCR assay was slightly but not significantly better than the probe-based qPCR assay, but the opposite happened in September, when samples tested with SYBR didn't show any positive reaction. For probes, the upper limit of the dynamic range is set at Cq 34,5 and for SYBR, Cq 32.

Figure 15 – Comparison of salmon detection in October 2020, Haugsdalselva, using probe based versus SYBR based qPCR assays. The Y axis shows the Quantification cycle from qPCR and the X axis is divided by sampling stations. Horizontal lines represent the LOD for each assay, with probe based having a threshold of 34.5 Cq and SYBR assay 32 Cq.
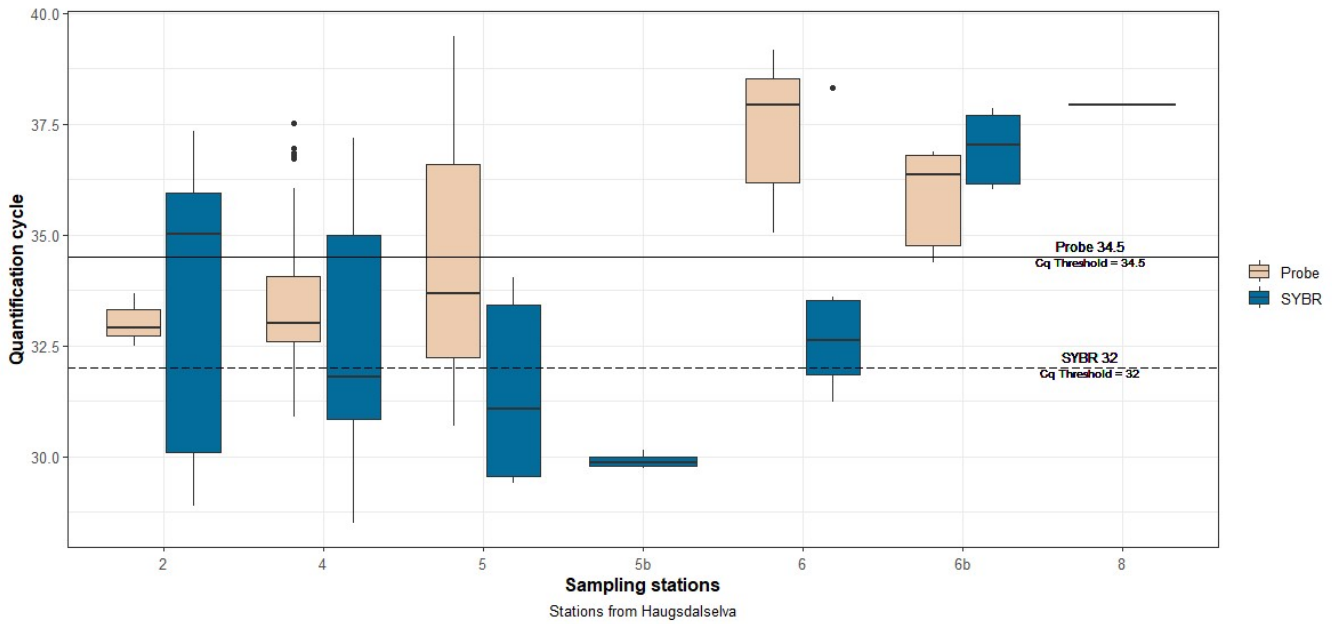
Table 7 – Detection of salmon eDNA from samples from Haugsdalselva in October tested in qPCR with SYBR green based assay and probe-based assays

Stations	Probe assay			SYBR assay		
	Station replicates	Average Cq and SD	Positive PCRs	Station replicates (%)	Average Cq and SD	Positive PCRs
2	3/5	33.11 ± 0.40	8/15	5/5	29.65 ± 0.64	15/15
4	5/5	32.72 ± 073	15/15	5/5	30.73 ± 0.83	15/15
5	2/3	31.85 ± 0.88	6/9	2/2	31.03 ± 2.23	4/8
5b	1/3	34,5	1/9	1/2	29.91 ±0.21	3/6
6b	0/5	-	0/15	0/5	-	

Table 8 - Comparison of Probe and SYBR assay for September in Haugsdalselva.

Stations	Probe assay			SYBR assay		
	Station replicates	Average Cq and SD	Positive PCRs	Station replicates (%)	Average Cq and SD	Positive PCRs
2	1/5	33.46 ± 0.9	3/15	0/8	-	0/24
4	1/5	33.27 ± 0.9	3/15	0/5	-	0/15
					-	
6b	1/5	34.25	1/15	0/5	-	0/15
					-	

6.6 Extraction methods influence in detection of Atlantic Salmon

In May, stations 2 and 4 detected Salmon with both qPCR and ddPCR, station 4 is shown to have the highest concentration of salmon DNA by lower Cq values among replicates and higher rates of detection in biological (3 out of 5 biological replicates) and technical (total of 9 detections out of 15 PCR runs), for samples conserved with PW1 and extracted with DNeasy PowerWater kit (Table 9).

One replicate out of 15 conserved with ATL had a positive detection, also in station 4. Four replicates of station 4 results were discarded due to abnormal amplification curve from qPCR. Three biological replicates from station 2 also detected salmon, with a rate of 7/15 total positive detections.

For station 8, 4 out of 15 replicates had an amplification outside the range of detection and for ddPCR, 6 replicates showed one positive droplet also below limit of detection.

As for ddPCR, three replicates from station 2 treated with DNeasy Blood and Tissue were positive, while with qPCR only one sample from station treated with ATL.

Table 9 – Atlantic salmon detection in Haugsdalselva in May, with probe assays for qPCR and ddPCR. Table is divided into stations 2 and 4, and extraction kits used, DNeasy Blood and Tissue and DNeasy Power Water kit. Limit of detection for the assay with qPCR is Cq 34.5 and table shows the range of Cq between positive replicates. For ddPCR, a positive detection is given when samples present concentration above 0.36 copies/uL and 4 droplets. Samples conserved in ethanol and extracted with BT kit and negative biological control samples (station 8) were negative and are not included in the table.

Month	Station	qPCR		ddPCR	
		Cq range	Positive detections	Copies/uL	Positive detections
May	2 PW	32.95 -33.85	7/15	0.41 to 1.8	9/15
	2 BT	-	0/15	0.33	1/15
	4 PW	33.21- 34.25	9/15	0.48 – 1.6	8/15
	4 BT	34.25	1/15	0.38 – 0.40	2/15

7. Discussion

This thesis aimed at comparing different methods, protocols, instruments and chemistries for efficiency and reliability at detecting Atlantic salmon DNA in Haugsdalselva and validating weather an eDNA based detection technique is congruent with previous drift-diving surveys conducted in the same site.

The results presented in this thesis shows eDNA detection of Atlantic salmon to be congruent with the spawning fish count survey conducted in 2020 by LFI, NORCE (Skoglund et al., 2020) and with the life cycle and migration patterns of Atlantic Salmon in the study site, demonstrating the suitability of this assay for species detection and its potential for future applications in monitoring.

Species distribution is a key factor in conservation and management, and a timely and cost effective tool such as eDNA for monitoring can not only completement traditional survey methods but compensate for its limitations with its high detection ability of cryptic and rare species, easy access of a great variety of habitats for sampling and for being cheaper and less labor intensive(Deiner et al., 2021; Fossøy et al., 2020; Sigsgaard et al., 2015; Yamanaka & Minamoto, 2016).

In this scenario, efforts in optimization and validation of best suited protocols for eDNA detection are crucial for broadening its application and reliability for monitoring.

7.1 DNA yield in environmental samples

There are several challenges for accurate detection and recovery of eDNA from samples and how to choose the correct protocols is an extremely important part of any eDNA study (Hunter et al., 2019; Wu & Minamoto, 2023; Xing et al., 2022), and here I compared two commercial available kits from Qiagen.

My study points out DNeasy Power Water kit® performs better than DNeasy Blood and Tissue when considering total DNA yield, accurate detection of my target species through PCR and removal of inhibitors that can be co-extracted with environmental samples.

Despite that most studies don't use water dedicated extraction kits for samples, examples from literature include QIAamp Tissue Extraction Kit (Qiagen) (Ficetola et al., 2008), DNeasy® Mericon Food Qiagen (Williams et al., 2016) and many studies using DNeasy Blood and Tissue also from Qiagen (Agersnap et al., 2017; Fossøy et al., 2020; Jacobsen et al., 2023; Kelly et al., 2014; Kirtane et al., 2021; Knudsen et al., 2019; Yamanaka & Minamoto, 2016).

One common argument against specific commercial extraction kits for water samples is the cost (Xing et al., 2022), for example PowerWater kit from Qiagen is approximately two and half times more expensive than Blood and Tissue. Reducing the cost may compromise sensibility, so evaluating study sites and conditions should be the parameter for kit choice. In cases where samples come from turbid waters or when species is known to occur in lower frequencies, the choice of a more robust method to deal with inhibition should be taken into consideration to tackle some of the challenges in total DNA yield and target species signal.

In my evaluation of water samples collected in May, Blood and Tissue kit performed much poorer than PowerWater, and the combination of ethanol and extraction with BT resulted in most of the samples not showing any amplification. Despite that, total DNA yield measured with QuBit didn't show any statistically significant difference in conservation with ATL or with Ethanol.

7.2 Inhibition

Presence of inhibitors are a common problem in eDNA studies or surveys as they can impact sensitivity of detection methods and increase the rate of false negatives. Inhibitors can promote DNA degradation, interfere with extraction, inhibit polymerase activity, bind to nucleic acids and prevent accurate quantification of a target DNA (Gibson et al., 2012; Hunter et al., 2019).

Presence of inhibitors seem to be higher in the summer in Haugsdalselva. Samples collected in the summer, August, showed the highest concentration in eDNA yield and yet qPCR assay for Salmon failed to detect salmon DNA in all but one replicate. The same didn't happen with ddPCR, that has gained space in eDNA research due to a better resistance against inhibitors than qPCR (Mauvisseau et al., 2019).

Tests with the spike assay in qPCR with samples from all seasons show a dramatic variation between the different seasons, with February showing least inhibition and more consistence of amplification between replicates.

Inhibition doesn't occur equally in samples and inhibitors can interact with DNA in unexpected ways. In one study on invasive plant species in water bodies by d'Auriac et al (2022), water turbidity caused by high levels of clay did not hamper eDNA detection, and despite being the cause of very low sample volume. One reason given is that DNA can bind to different particles or minerals and become more resistant to degradation.

7.3 Dilution

Despite not having an exclusive step for removal of inhibitors, when diluted DNeasy Blood and Tissue showed similar results in the qPCR runs for *H. salinarum* assay to the PowerWater kit (Figure 13), though diluting samples could potentially result in false negatives due to the typically low DNA concentrations in environmental samples, as also seen in table 8 with qPCR results for samples collected in May with DNeasy Power Water and Blood and Tissue. Samples conserved in ethanol didn't detect salmon.

I decided to treat all my samples in 2-fold dilutions before PCR analysis with the goal of reducing potential inhibition and for archival purposes.

I tested samples from October (Table 6) to evaluate the dilution effect in Atlantic salmon detection against samples that didn't receive any sort of pre-treatment post extraction. Samples diluted 2:1 had a higher rate of positive PCR runs than undiluted samples.

Presence of inhibitors is a common constraint when handling eDNA samples and a common practice is to use dilutions. Despite its effectivity, this practice decreases sensitivity of the assay, mainly in samples where the target is present in very low concentrations (McKee et al., 2015). McKee et al. (2015) compared the effects of dilution 5-fold, 10-fold and a spin-column purification kit and shown they were equally effective reducing PCR inhibitors in water samples collected from mountain headwater streams.

In fact, as seen in Fig.14, 5-fold dilution didn´t differ greatly from 10-fold dilution on qPCR detections with the spike assay, and even DNeasy Blood and Tissue extracted samples were able to show amplification with the spike (*H. salinarum*) assay after dilutions.

Some studies, though, have not seen any additional positive detection from diluted samples compared with undiluted ones, like a study by Sigsgaard et al (2015), where samples with no initial amplification were diluted into 1:10 in ddH2O for re-analysis. This is probably due to dilution of the target DNA causing it to fall below levels of detectability.

7.4 PCR quantification and specificity

The cut-off value for specificity for this assay shows a dramatic difference if samples were to be compared with our limit of detection. For qPCR, our limit of detection was set at Cq 36 and for ddPCR, 0.36 copies/uL and 4 droplets. The manufacturer manual states that 0.25 copies/uL and 3 droplets are the lower end of the limit of detection, which we decided to not adopt as unspecific amplification happened within the parameters above.

Despite similar performances overall in other months, ddPCR managed to detect Atlantic salmon DNA in August in most of the replicates, which is coherent with the current attention it has been gaining in eDNA studies for being more tolerant to the effects of PCR inhibitors.

Another reason for its use is the precision in quantification: techniques based on ddPCR are regarded as more precise as the quantification does not require a standard curve for calibration and relative measurements, doesn´t depend on reaction efficiency and have been shown to be more reproducible across laboratories.

One advantage of using qPCR, though, is its dynamic range. In my comparison, I showed that the lower limit of the LOD (limit of detection), is similar for both PCR instruments (Figures 5, 6 and 7). But qPCR overperforms ddPCR, as it can reach 6 orders of magnitude (The dMIQE Group et al., 2020) – which, for environmental samples, doesn't necessarily offer an advantage.

To perform an analysis, ddPCR requires more consumable accessories and extra steps when compared with qPCR. Besides the well plates, for a ddPCR run one needs droplet generation oils, droplet reader oil, cartridges for droplet generation, gaskets, and a cartridge holder. This could make the choice of using ddPCR less attractive or and economical constraint, and using ddPCR it would depend on the resources available for a study or survey.

7.5 Salmon detection comparison with drift diving

The assay was successful in detection Atlantic salmon in all marked stations (2, 4, 5 and 6) across the year (Figure 14) but station number 8, our biological negative control above migration barrier. Detection was also consistent with drift diving surveys and migration pattern.

Highest number of detections per replicate is found on stations 2 (around site 15) and 4 (around site 10).

Salmon was detected by conventional fish counting methodology in Haugsdalselva by NORCE LFI (Skoglund et al., 2021). All salmon was observed in part of the rivers numbered as river section 8 and

10 (see Figure 17). These river sections correspond to Station 5 and Station 4 in the present study. Previous surveys have also identified area in river section 8 as the spawning ground (Skoglund et al 2020). It was also observed that Atlantic salmon can swim up to around Station 6 and is not seen above station 8 (migration barrier).

Station 2 is located downstream, which could be one reason to which it shows positive results during all seasons. Several studies tested eDNA mobility across water bodies and persistence (Debes et al., 2017; Deiner et al., 2021) and a study from Tillotson et al. 2018 using probe assay for salmon detection has shown that eDNA seemed to be conserved over short distances and past the mark of 1.5 km it would not be detectable. The distance between the spawning area from the mentioned report and my sampling station number 2 is 1.4 km.
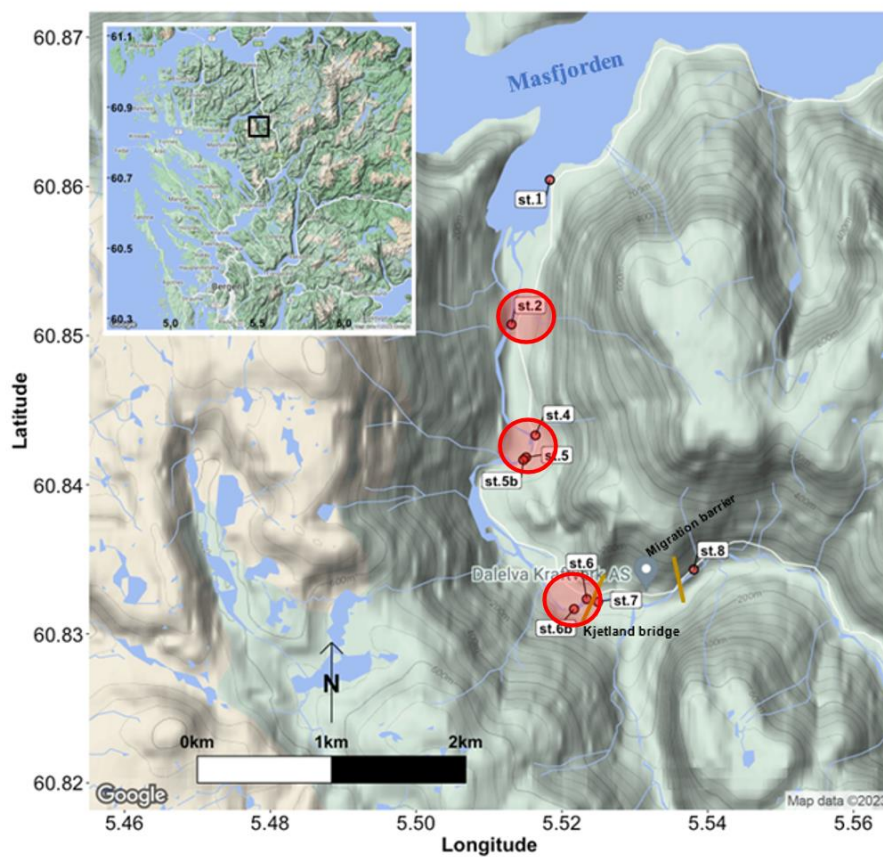

Figure 16 – Positive detection of salmon in Haugsdalselva in 2020. Stations 2, 4, 5 and 6 tested with qPCR presence of salmon. Station 4 has the highest percentage of positive technical and biological replicates.
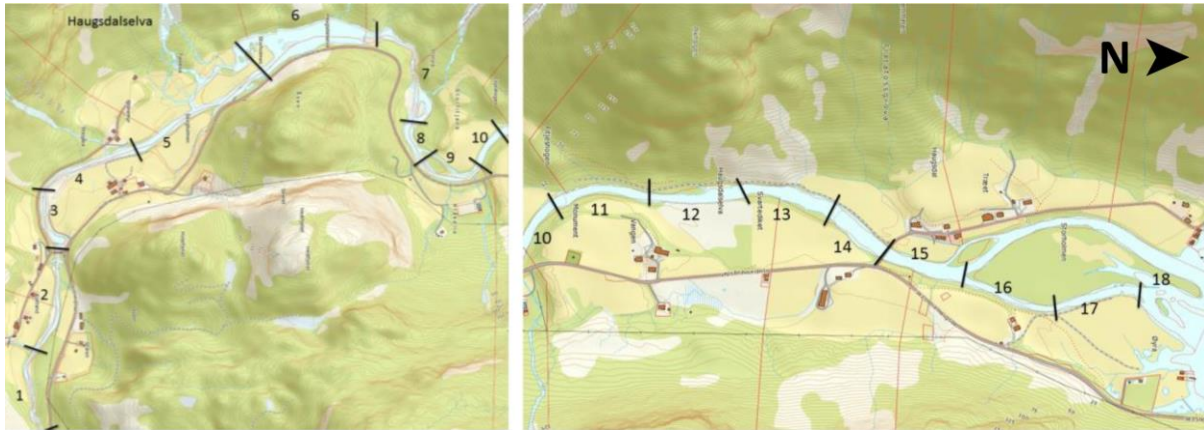
Figure 17 – Spawning fish counting conducted in Haugsdalselva in October 2020 by NORCE LFI (Skoglund et al 2021) detected salmon in river sections 8 and 10 in the above map. Site 8 in the figure corresponds to Station 5 and site 10 is in proximity of station 4 in this study. Figure is from NORCE LFI report no. 401 (Skoglund et al 2021).

In May, stations 2 and 4 detected Salmon with both qPCR and ddPCR, station 4 is shown to have the highest concentration of salmon DNA by lower Cq values among replicates and higher rates of detection in biological (3 out of 5 biological replicates) and technical (total of 9 detections out of 15 PCR runs), for samples conserved with PW1 and extracted with DNeasy PowerWater kit.

One replicate out of 15 conserved with ATL had a positive detection, also in station 4. Four replicates of station 4 results were discarded due to abnormal amplification curve output from qPCR. Three biological replicates from station 2 also detected salmon, with a rate of 7/15 total positive detections.

For ddPCR, four total replicates from station 2 treated with DNeasy Blood and Tissue using ATL as conservation buffer were positive for salmon, three more than qPCR for this kit.

Fish counting in Haugsdalselva takes place during spawning season, October, when a higher number of fish is expected. This result is also reflected on qPCR assays comparison between samples from other months and samples taken during spawning season; more replicates were positive in October.

8. Conclusions

This thesis assessed and compared different methods and protocols for detection of Atlantic Salmon DNA in Haugsdalselva, Norway. There is a high congruence between eDNA detection in freshwater samples from 2020 to the spawning fish counting survey conducted the same year.

Considering total DNA yield and removal of PCR inhibitors, the DNeasy Power Water extraction kit performed dramatically better than the Blood and Tissue kit, tested for inhibition with the spike PCR assay and for Atlantic salmon detection with both qPCR and ddPCR assays. As for instruments, ddPCR performed slightly better than qPCR in May for station 2 and was markedly better at detection in samples from Summer.

Previous surveys have identified areas around Station 2 and 4 to have highest density of juvenile salmonids, which is also compatible to eDNA detection with most protocols tested.

Presence and absence of eDNA target was also consistent with known patterns of life history and seasonal migration, with discreet eDNA detection during winter and higher detection during spawning season.

While there is a need for better understanding of the dynamics affecting eDNA degradation, inhibition and relation to biomass, the tools used in this thesis have been proven to have great potential that could be used in ecological studies and surveys as they are cost effective, simple, and fast. They could also be an important supplement to compensate drawbacks and methodological difficulties of other traditional monitoring techniques.

9. References

Agersnap, S., Larsen, W. B., Knudsen, S. W., Strand, D., Thomsen, P. F., Hesselsøe, M., Mortensen, P. B., Vrålstad, T., & Møller, P. R. (2017). Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PLOS ONE*, *12*(6), e0179261. https://doi.org/10.1371/journal.pone.0179261

Ahmed, S., Ibrahim, M., Nantasenamat, C., Nisar, M. F., Malik, A. A., Waheed, R., Ahmed, M. Z., Ojha, S. C., & Alam, M. K. (2022). Pragmatic Applications and Universality of DNA Barcoding for Substantial Organisms at Species Level: A Review to Explore a Way Forward. *BioMed Research International*, *2022*, e1846485. https://doi.org/10.1155/2022/1846485

Andersen, L., Hodneland, K., & Nylund, A. (2010). No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (Salmo salar L.). *Virology Journal*, *7*(1), Article 1. https://doi.org/10.1186/1743-422X-7-198

Atkinson, S., Carlsson, J. E. L., Ball, B., Egan, D., Kelly-Quinn, M., Whelan, K., & Carlsson, J. (2018). A quantitative PCR-based environmental DNA assay for detecting Atlantic salmon (*Salmo salar* L.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *28*(5), 1238–1243. https://doi.org/10.1002/aqc.2931

Bio-Rad. (n.d.). *Droplet Digital^{TM} PCR Applications Guide. Bulletin 6407*.

Bolstad, G. H., Hindar, K., Robertsen, G., Jonsson, B., Sægrov, H., Diserud, O. H., Fiske, P., Jensen, A. J., Urdal, K., Næsje, T. F., Barlaup, B. T., Florø-Larsen, B., Lo, H., Niemelä, E., & Karlsson, S. (2017). Gene flow from domesticated escapes alters the life history of wild Atlantic salmon. *Nature Ecology & Evolution*, *1*(5), Article 5. https://doi.org/10.1038/s41559-017-0124

Buckwalter, S. P., Sloan, L. M., Cunningham, S. A., Espy, M. J., Uhl, J. R., Jones, M. F., Vetter, E. A., Mandrekar, J., Cockerill, F. R., Pritt, B. S., Patel, R., & Wengenack, N. L. (2014). Inhibition Controls for Qualitative Real-Time PCR Assays: Are They Necessary for All Specimen Matrices? *Journal of Clinical Microbiology*, *52*(6), 2139–2143. https://doi.org/10.1128/JCM.03389-13

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Dalvin, S., Glover, K. A., Sørvik, A. G., Seliussen, B. B., & Taggart, J. B. (2010). Forensic identification of severely degraded Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) tissues. *Investigative Genetics*, *1*(1), 12. https://doi.org/10.1186/2041-2223-1-12

Davidson, W. S., Koop, B. F., Jones, S. J., Iturra, P., Vidal, R., Maass, A., Jonassen, I., Lien, S., & Omholt, S. W. (2010). Sequencing the genome of the Atlantic salmon (Salmo salar). *Genome Biology*, *11*(9), 403. https://doi.org/10.1186/gb-2010-11-9-403

Debes, P. V., Gross, R., & Vasemägi, A. (2017). Quantitative Genetic Variation in, and Environmental Effects on, Pathogen Resistance and Temperature-Dependent Disease Severity in a Wild Trout. *The American Naturalist*, *190*(2), 244–265. https://doi.org/10.1086/692536

Deiner, K., Yamanaka, H., & Bernatchez, L. (2021). The future of biodiversity monitoring and conservation utilizing environmental DNA. *Environmental DNA*, *3*(1), 3–7. https://doi.org/10.1002/edn3.178

Eichmiller, J. J., Miller, L. M., & Sorensen, P. W. (2016). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular Ecology Resources*, *16*(1), 56–68. https://doi.org/10.1111/1755-0998.12421

Evans, N. T., Shirey, P. D., Wieringa, J. G., Mahon, A. R., & Lamberti, G. A. (2017). Comparative Cost and Effort of Fish Distribution Detection via Environmental DNA Analysis and Electrofishing. *Fisheries*, *42*(2), 90–99. https://doi.org/10.1080/03632415.2017.1276329

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification*, *12*, 1–6. https://doi.org/10.1016/j.bdq.2017.04.001

Forseth, T., Barlaup, B. T., Finstad, B., Fiske, P., Gjøsæter, H., Falkegård, M., Hindar, A., Mo, T. A., Rikardsen, A. H., Thorstad, E. B., Vøllestad, L. A., & Wennevik, V. (2017). The major threats to Atlantic salmon in Norway. *ICES Journal of Marine Science*, *74*(6), 1496–1513. https://doi.org/10.1093/icesjms/fsx020

Fossøy, F., Brandsegg, H., Sivertsgård, R., Pettersen, O., Sandercock, B. K., Solem, Ø., Hindar, K., & Mo, T. A. (2020). Monitoring presence and abundance of two gyrodactylid ectoparasites and their salmonid hosts using environmental DNA. *Environmental DNA*, *2*(1), 53–62. https://doi.org/10.1002/edn3.45

Frazer, L. N. (2009). Sea-Cage Aquaculture, Sea Lice, and Declines of Wild Fish. *Conservation Biology*, *23*(3), 599–607. https://doi.org/10.1111/j.1523-1739.2008.01128.x

Gibson, K. E., Schwab, K. J., Spencer, S. K., & Borchardt, M. A. (2012). Measuring and mitigating inhibition during quantitative real time PCR analysis of viral nucleic acid extracts from large-volume environmental water samples. *Water Research*, *46*(13), 4281–4291. https://doi.org/10.1016/j.watres.2012.04.030

Gillson, J. P., Bašić, T., Davison, P. I., Riley, W. D., Talks, L., Walker, A. M., & Russell, I. C. (2022). A review of marine stressors impacting Atlantic salmon Salmo salar, with an assessment of the major threats to English stocks. *Reviews in Fish Biology and Fisheries*, *32*(3), 879–919. https://doi.org/10.1007/s11160-022-09714-x

Gorokhova, E. (2005). Effects of preservation and storage of microcrustaceans in RNA later on RNA and DNA degradation. *Limnology and Oceanography: Methods*, *3*. https://doi.org/10.4319/lom.2005.3.143

Hansen, H., Amundsen, M. M., Mohammad, S. N., & Allan, D. (n.d.). *A surveillance programme to document absence of Atlantic salmon (Salmo salar) and G. salaris in the River Drammenselva upstream of Hellefossen.*

Harvey, A. C., Glover, K. A., Wennevik, V., & Skaala, Ø. (2020). Atlantic salmon and sea trout display synchronised smolt migration relative to linked environmental cues. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-60588-0

Hunter, M. E., Ferrante, J. A., Meigs-Friend, G., & Ulmer, A. (2019). Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Scientific Reports*, *9*(1), 5259. https://doi.org/10.1038/s41598-019-40977-w

Jacobsen, M. W., Nygaard, R., Hansen, B. K., Broberg, M., Hansen, M. M., Hedeholm, R., & Nielsen, E. E. (2023). Observing the Arctic: A comparison of environmental DNA (EDNA) and electrofishing for monitoring Arctic char and Atlantic salmon. *Environmental DNA*, *5*(4), 782–795. https://doi.org/10.1002/edn3.442

Jonsson, B., & Jonsson, N. (2009). A review of the likely effects of climate change on anadromous Atlantic salmon *Salmo salar* and brown trout *Salmo trutta* , with particular reference to water temperature and flow. *Journal of Fish Biology*, *75*(10), 2381–2447. https://doi.org/10.1111/j.1095-8649.2009.02380.x

Jonsson, B., & Jonsson, N. (2011a). Climatic Effects on Atlantic Salmon and Brown Trout. In B. Jonsson & N. Jonsson (Eds.), *Ecology of Atlantic Salmon and Brown Trout: Habitat as a template for life histories* (pp. 473–515). Springer Netherlands. https://doi.org/10.1007/978-94-007-1189-1_9

Jonsson, B., & Jonsson, N. (2011b). *Ecology of Atlantic Salmon and Brown Trout: Habitat as a template for life histories*. Springer Netherlands. https://doi.org/10.1007/978-94-007-1189-1

Kelly, R. P., Port, J. A., Yamahara, K. M., & Crowder, L. B. (2014). Using Environmental DNA to Census Marine Fishes in a Large Mesocosm. *PLoS ONE*, *9*(1), e86175. https://doi.org/10.1371/journal.pone.0086175

Kirtane, A., Wieczorek, D., Noji, T., Baskin, L., Ober, C., Plosica, R., Chenoweth, A., Lynch, K., & Sassoubre, L. (2021). Quantification of Environmental DNA (eDNA) shedding and decay rates for three commercially harvested fish species and comparison between eDNA detection and trawl catches. *Environmental DNA*, *3*(6), 1142–1155. https://doi.org/10.1002/edn3.236

Knudsen, S. W., Ebert, R. B., Hesselsøe, M., Kuntke, F., Hassingboe, J., Mortensen, P. B., Thomsen, P. F., Sigsgaard, E. E., Hansen, B. K., Nielsen, E. E., & Møller, P. R. (2019). Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. *Journal of Experimental Marine Biology and Ecology*, *510*, 31–45. https://doi.org/10.1016/j.jembe.2018.09.004

Kumar, G., Eble, J. E., & Gaither, M. R. (2020). A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. *Molecular Ecology Resources*, *20*(1), 29–39. https://doi.org/10.1111/1755-0998.13107

Liu, Y., Olaf Olaussen, J., & Skonhoft, A. (2011). Wild and farmed salmon in Norway—A review. *Marine Policy*, *35*(3), 413–418. https://doi.org/10.1016/j.marpol.2010.11.007

Mahlum, S. K., Vollset, K. W., Barlaup, B. T., Velle, G., & Wiers, T. (2018). Where the salmon roam: Fjord habitat use of adult Atlantic salmon. *ICES Journal of Marine Science*, *75*(6), 2163–2171. https://doi.org/10.1093/icesjms/fsy071

Mahlum, S., Skoglund, H., Wiers, T., Norman, E., Barlaup, B., Wennevik, V., Glover, K., Urdal, K., Bakke, G., & Vollset, K. (2019). Swimming with the fishes: Validating drift diving to identify farmed Atlantic salmon escapees in the wild. *Aquaculture Environment Interactions*, *11*, 417–427. https://doi.org/10.3354/aei00326

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., & Sweet, M. (2019). Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered

freshwater invertebrate. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-50571-9

McColl-Gausden, E. F., Weeks, A. R., Coleman, R. A., Robinson, K. L., Song, S., Raadik, T. A., & Tingley, R. (2021). Multispecies models reveal that eDNA metabarcoding is more sensitive than backpack electrofishing for conducting fish surveys in freshwater streams. *Molecular Ecology*, *30*(13), 3111–3126. https://doi.org/10.1111/mec.15644

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation*, *183*, 70–76. https://doi.org/10.1016/j.biocon.2014.11.031

Øystein Aas, Anders Klemetsen, Sigurd Einum, & Jostein Skurdal. (2010). *Atlantic Salmon Ecology* (1st edition). Wiley-Blackwell.

Roberge, C., Normandeau, É., Einum, S., Guderley, H., & Bernatchez, L. (2008). Genetic consequences of interbreeding between farmed and wild Atlantic salmon: Insights from the transcriptome. *Molecular Ecology*, *17*(1), 314–324. https://doi.org/10.1111/j.1365-294X.2007.03438.x

Rourke, M. L., Fowler, A. M., Hughes, J. M., Broadhurst, M. K., DiBattista, J. D., Fielder, S., Wilkes Walburn, J., & Furlan, E. M. (2022). Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA*, *4*(1), 9–33. https://doi.org/10.1002/edn3.185

Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, *183*, 46–52. https://doi.org/10.1016/j.biocon.2014.11.023

Skoglund, H., Vollset, K. W., Lennox, R., Skaala, Ø., & Barlaup, B. T. (2021). Drift diving: A quick and accurate method for assessment of anadromous salmonid spawning populations. *Fisheries Management and Ecology*, *28*(5), 478–485. https://doi.org/10.1111/fme.12491

Skoglund, H., Wiers, T., Normann, E., Landro, Y., & Postler, C. (2020). *Gytefisktelling i BKK-regulerte elver høsten 2020* (401; LFI - Laboratorium for ferskvannsøkologi og innlandsfiske). NORCE Norwegian Research Centre. https://hdl.handle.net/11250/2758588

Snyder, D. E. (2003). Invited overview: Conclusions from a review of electrofishing and its harmful effects on fish. *Reviews in Fish Biology and Fisheries*, *13*(4), 445–453. https://doi.org/10.1007/s11160-004-1095-9

Stewart, K. A. (2019). Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. *Biodiversity and Conservation*, *28*(5), 983–1001. https://doi.org/10.1007/s10531-019-01709-8

Strickland, G. J., & Roberts, J. H. (2019). Utility of eDNA and occupancy models for monitoring an endangered fish across diverse riverine habitats. *Hydrobiologia*, *826*(1), 129–144. https://doi.org/10.1007/s10750-018-3723-8

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

The dMIQE Group, Whale, A. S., De Spiegelaere, W., Trypsteen, W., Nour, A. A., Bae, Y.-K., Benes, V., Burke, D., Cleveland, M., Corbisier, P., Devonshire, A. S., Dong, L., Drandi, D., Foy, C. A., Garson, J. A., He, H.-J., Hellemans, J., Kubista, M., Lievens, A., … Huggett, J. F. (2020). The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. *Clinical Chemistry*, *66*(8), 1012–1029. https://doi.org/10.1093/clinchem/hvaa125

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., … Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*(4), 929–942. https://doi.org/10.1111/mec.13428

Webb, J., Verspoor, E., Aubin-Horth, N., Romakkaniemi, A., & Amiro, P. (2007). The Atlantic Salmon. In E. Verspoor, L. Stradmeyer, & J. Nielsen (Eds.), *The Atlantic Salmon* (pp. 17–56). Blackwell Publishing Ltd. https://doi.org/10.1002/9780470995846.ch2

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *PLoS ONE*, *8*(3), e59520. https://doi.org/10.1371/journal.pone.0059520

Williams, K. E., Huyvaert, K. P., & Piaggio, A. J. (2016). No filters, no fridges: A method for preservation of water samples for eDNA analysis. *BMC Research Notes*, *9*(1), 298. https://doi.org/10.1186/s13104-016-2104-5

Wu, Q., & Minamoto, T. (2023). Improvement of recovery yield of macro-organismal environmental DNA from seawater samples. *Analytical Sciences*, *39*(5), 713–720. https://doi.org/10.1007/s44211-023-00280-1

Xing, Y., Gao, W., Shen, Z., Zhang, Y., Bai, J., Cai, X., Ouyang, J., & Zhao, Y. (2022). A Review of Environmental DNA Field and Laboratory Protocols Applied in Fish Ecology and Environmental Health. *Frontiers in Environmental Science*, *10*, 725360. https://doi.org/10.3389/fenvs.2022.725360

Yamanaka, H., & Minamoto, T. (2016). The use of environmental DNA of fishes as an efficient method of determining habitat connectivity. *Ecological Indicators*, *62*, 147–153. https://doi.org/10.1016/j.ecolind.2015.11.022

APPENDIX

Table A – DNA extraction and Qubit dsDNA HS (High Sensitivity) assay kits

Name	Use	Catalogue Nr.	Producer
DNeasy Blood and Tissue	DNA Extraction and purification kit for Blood and Tissue	69504	Qiagen
DNeasy PowerWater	DNA Extraction and Purification for Aquatic Environmental Samples	14900-100-NF	Qiagen
dsDNA QuBit High Sensitivity	Non-specific DNA concentration quantification	Q33230	Thermofisher

Table B – PCR assays; Primers and probes used for targeting Atlantic salmon (*Salmo salar*) and *Halobacterium salinarum* used as an exogenous control (spike)

	Sequence 5'-3'		Producer	Reference number
Salmo salar L. Atkinson et.al (2018)	CGC CCT AAG TCT CTT GAT TCG A	Forward Primer	Sigma-Aldrich	8813717766-0000010
	TAT AAA TTT GGT CAT CTC CCA GA	Reverse Primer	Sigma-Aldrich	8813717766-0000020
	5′-NED-AGA ACT CAG CCA GCC TG-3′	Probe 5'-3'	Sigma-Aldrich	8815372374
Halobacterium salinarum Andersen et al. (2010)	[6FAM]AGGCGTCCAGCGGA[BHQ1]	Probe	Sigma-Aldrich	8812248280-000010
	GGGAAATCTGTCCGCTTAACG	Forward Primer	Sigma Aldrich	8812248280-000020
	CCGGTCCCAAGCTGAACA	Reverse Primer	Sigma Aldrich	8812248280-000030

Table C – Commercial solutions used for preservation of samples and PCR (qPCR, SYBR Green and ddPCR) reactions

Solution name	Producer	Catalogue Nr.
Droplet Generation Oil for Probes	BioRad	1863005
ddPCR supermix for probes (No dUTP)	BioRad	1863024
QX200™ ddPCR™ EvaGreen Supermix	BioRad	186-4033
Droplet Generation Oil for EvaGreen	BioRad	1864005
SsoAdvanced Universal Probes Supermix	BioRad	172-5280
SsoAdvanced Universal SYBR Green Supermix	BioRad	1725270
Tris Buffer, 1.0 M, pH 8.0, Molecular Biology Grade - CAS 77-86-1	Merckmillipore	648314
ATL Tissue Lysis Buffer for purification of nucleic acids	Qiagen	939016

Table D – Equipment used in laboratory for processing of the water samples.

Equipment	Catalog Number	Producer
QuBit 4 Fluorometer	Q33239	Thermofisher Scientific Inc
QX200 Droplet Digital PCR Reader	1864003	Bio-Rad
PX1 PCR Plate Sealer	1814000	Bio-Rad
QX200 Droplet Generator	1864002	Bio-Rad
CFX96 Optical Reaction Module for Real-Time PCR	1845097	Bio-Rad
C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module #	1851197	Bio-Rad
Vacuum Filtration System 6 chambers 0,5 L	22.050	KC Denmark
Water tank for filtration station	22.051	KC Denmark
Vacuum Pump	22.029	KC Denmark
TissueRuptor II	9002756	Qiagen
TissueRuptor Disposable Probes (25)	990890	Qiagen