University of South-Eastern Norway

Faculty of Technology, Natural Sciences and Maritime Sciences
Master's Thesis
Study programme: Ecology & Environmental Management
Spring 2024

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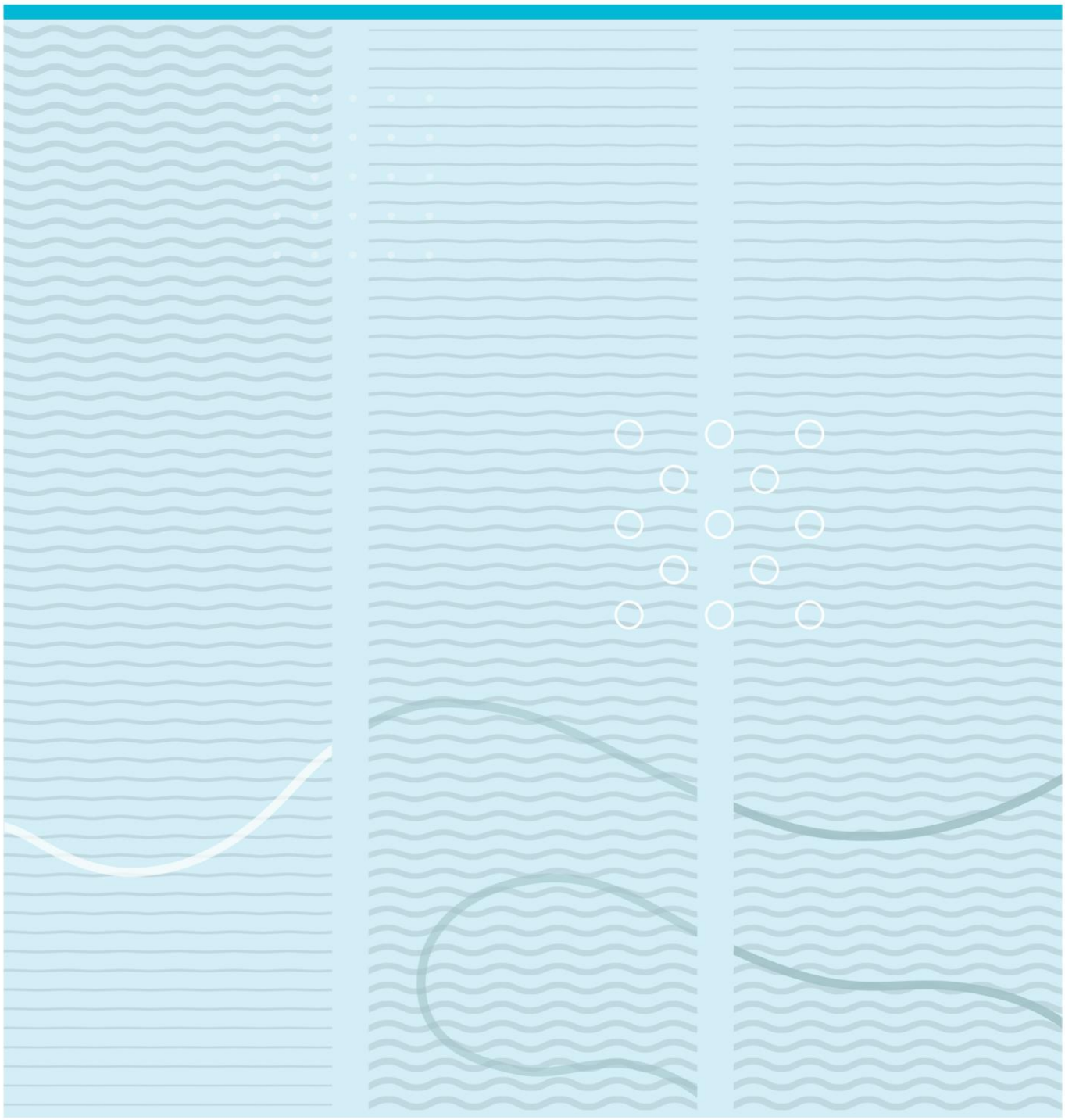
Investigating the presence of *Tetracapsuloides bryosalmonae* in River Sandvikselva by eDNA analysis



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This thesis is worth 60 study points

Abstract

Tetracapsuloides bryosalmonae is a myxozoan parasite that poses a threat to salmonid fish populations leading to development of the potential lethal Proliferative Kidney Disease (PKD). The presence and effects of *T. bryosalmonae* has been studied through many surveys in Europe and in Norway with traditional methods and eDNA analysis over the last decades.

Optimizing study design in eDNA analysis is important to reduce detection errors and optimize detection reliability in both sampling and analysis stages. The advantage of using eDNA in ecological studies is that it is a non-invasive method, compared to the invasive sampling and killing the host to detect a potential hidden parasite. The myxozoan parasite is primarily found in rivers and lakes inside their salmonid and bryozoan hosts, where the host morbidity is often exacerbated by climate change and warmer temperatures.

This master-thesis aim to investigate River Sandvikselva for the presence of *T. bryosalmonae* by eDNA analysis during the summer months 2023. Water sampling was done using a Peristaltic pump and an enclosed filter to sample 10 L of water. For DNA extraction a protocol for isolation of DNA from water-filter to eDNA analysis, and a modified Qiagen Blood and Tissue-kit was used. To detect *T. bryosalmonae* qPCR was used targeting the 18S rDNA.

T. bryosalmonae presence was detected in River Sandvikselva in station 1 and station 2, the two stations in the lower part of the watercourse. In station 1, four out of four samples tested positive in Juli and August, whilst in station 2 only one of four samples tested positive suggesting that *T. bryosalmonae* has been established in the lower part of River Sandvikselva. Additionally, the results highlight a low sensitivity that may have contributed to limiting the detection rate in the upper stations.

Contents

Preface

Undertaking this study has been a privilege and a challenge that have better shaped my understanding of the myxozoan parasite *Tetracapsuloides bryosalmonae* and use of environmental DNA. The journey documented in this master-thesis represents countless hours reading articles, working in the field and in the lab. This master-thesis not only reflect my work, but also the invaluable guidance from my supervisor Tone Jøran Oredalen, an incredible kind and knowledgeable teacher. I must also extend my gratitude to supervisor Tor-Atle Mo from the Norwegian Institute for Nature Research (NINA) for his expertise in sampling and valuable knowledge in this field. I also want to thank NINA for contributing financially and letting me be a part of their project. I want to thank Prof. Andrew Jenkins for his help and guidance in the laboratory.

Lastly, I want to thank my family and friends for their unwavering mental support during hard times. I am thankful for my friends Jens Even Vøllestad, Marte Bottolfs Brekke, Haris Khan and Eduard Codó Cónsol who were able to lend me their cars and join me for sampling.

Bø i Telemark, 07/05/2024

Vietphong Le Ton

Introduction

Environmental DNA (eDNA) techniques are increasingly utilized within aquatic environments to detect species and monitor biodiversity in freshwater ecosystems (Rishan et al., 2023). eDNA refers to genetic material from cells, mucus or skin extracted from the environmental samples, which can come from soil, air or water (Taberlet et al., 2012). eDNA is a non-invasive method and is often easier, more cost-effective, and more sensitive compared to traditional sampling methods (Fediajevaite et al., 2021).

In eDNA research, it is important to optimize the study design to reduce detection errors throughout both sampling and analysis stages (Buxton et al., 2021; Spens et al., 2017). Two types of major detection errors can be distinguished, false positives and false negatives (Burian et al., 2021). To reduce these sampling errors in the field, one should consider the most effective sampling strategy, under which circumstances contaminations can occur and how to prevent them (Carraro et al., 2021). For optimal sampling, it is important to consider the ecology of the target organism and sampling volume. Sampling volume should be adapted to the characteristics of the water body (Rees et al., 2014), and sample replication should also be considered to ensure detection reliability and reduce errors in both sampling and analysis stages (Buxton et al., 2021).

For more effective sampling, optimal technologies should be considered, such as the benefits of enclosed filters and peristaltic pumps (Patin & Goodwin, 2023). The benefits of enclosed filters include reduction in contamination and fast filtering time. By filtering in the field, the influences of DNA-degradation when travelling from sampling site to the lab is reduced (Patin & Goodwin, 2023).

The detection rate depends on the density and distribution of the target species in the water body (Carraro et al., 2018; Dejean et al., 2011) and the spatial heterogeneity of eDNA transport (Wood et al., 2021). Additionally, environmental factors can also influence the

detection. These factors include, pH, temperature, UV-radiation, water flow, presence of inhibitors, and other chemicals that persist or degrade the DNA, or influence target species (Jane et al., 2015; Joseph et al., 2022; Strokal et al., 2021)

In the laboratory, one should consider the most effective DNA extraction protocol and qPCR protocol to reduce potential detection errors (Spens et al., 2017). Factors such as the purity and the quality of the DNA samples, as well as the extraction process can significantly impact the accuracy of downstream analysis (Spens et al., 2017). Additionally, it is essential to consider any inhibitors of DNA amplification such as contaminants or substances present in the sample (Hunter et al., 2019). Furthermore, evaluating the sensitivity of the tests to ensure reliable detection of the target DNA, particularly when dealing with low abundances of target species (Furlan et al., 2016; Stelzer et al., 2024). By understanding the ecological dynamics of the target species under study, we are more likely to detect them using the optimal methods.

Tetracapsuloides bryosalmonae, a myxozoan parasite that poses a threat to salmonid fish leading to the development of Proliferative Kidney Disease (PKD). In Europe *T. bryosalmonae* and PKD have been surveyed extensively for the last decades. In Swiss rivers different methods have been used, including electrofishing (Rubin et al., 2019) and by eDNA analysis over large areas (Sieber et al., 2023). In Germany, they studied current and expected effects on salmonid populations due to *T. bryosalmonae* and climate change (Ros et al., 2021). Additionally, a survey from Estonia investigated the Baltic Sea for PKD in fish by rod-and-reel type fishing (Lauringson et al., 2023). In England they have surveyed *T. bryosalmonae* by collecting and dissecting bryozoans (Fontes et al., 2017a; Okamura et al., 2011).

In Norway *T. bryosalmonae* has been investigated extensively by net-fishing and electrofishing in rivers with subsequent analyses of fish kidneys (Mo & Jørgensen, 2017), by analysis the presence of the parasite in bryozoan colonies (Bendixby & Hals, 2009) and from

parasite DNA presence in water samples (eDNA) in rivers (Skrutvold & Roseth, 2023) and in deep Norwegian lakes (Oredalen et al., 2022).

Traditional sampling methods uses more invasive sampling involving killing of fish before examining the tissue (Mo & Jørgensen, 2017). This procedure is relatively time-consuming, labour intensive and expensive (Beng & Corlett, 2020). The eDNA method is a less invasive method, as it allows for monitoring of aquatic systems without capturing and killing host species, and it may detect species that are threatened or difficult to find due to low abundance in large areas (Sieber et al., 2023).

T. bryosalmonae depends on freshwater bryozoans and salmonid as hosts to complete its life cycle (Grabner & El-Matbouli, 2008; Morris & Adams, 2006).

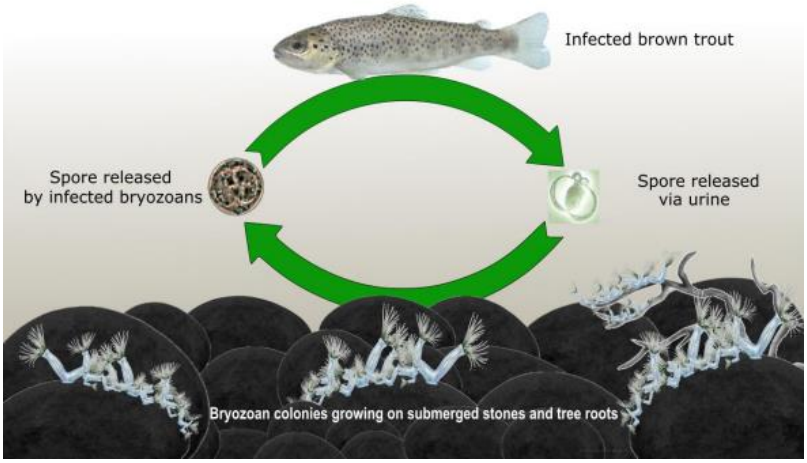

Figure 1. Life cycle of Tetracapsuloides bryosalmonae between the bryozoan and salmonid host. Figure from (Sudhagar et al., 2020)

Freshwater bryozoans often establish their colonies submerged in rivers and lakes on firm substrates (Økland & Økland, 2001). They are sessile, colonial invertebrates, where the individual zooid is often branched, elongated and they use their ciliated tentacles to capture phytoplankton and organic particles as filter-feeders (Økland & Økland, 2005). Bryozoans

normally thrive in nutrient rich conditions (Hartikainen et al., 2009) and in organically polluted waters (Thorp & Rogers, 2011). Bryozoans can reproduce asexually with their statoblast and through fission (Thorp & Rogers, 2011). They can also produce new zooids by sexual budding.

The investigated freshwater bryozoans in Norway include *Fredericella sultana*, *Cristatella mucedo* and *Plumatella* spp. (Økland & Økland, 2005; Økland & Økland, 2001; Økland et al., 2003). In a survey by Bendixby & Hals (2009) in River Åelva, Norway *T. bryosalmonae* is most found in *Fredericella sultana* compared to *Cristatella mucedo and Plumatella* spp though the latter two are more abundant in River Åelva (Bendixby & Hals, 2009).

In bryozoans, infections develop as single cells of parasite spore attaches to the bryozoan and spread into spore-producing sacs inside the bryozoan colonies (Morris & Adams, 2006). The colonies of bryozoans are at risk of infection of *T. bryosalmonae* when the water temperature rises over 14°C (Tops et al., 2009). Throughout the year, *T. bryosalmonae* can switch between covert and overt infection phases (Fontes et al., 2017b). Covert phases are defined by Inês Fontes (2017b) and is non-infective persistent forms of parasitic infections that undergoes latent or slow replication. When alternating from covert to overt infections the host starts exhibiting symptoms, and sets unstable energy demands on the bryozoan host, reducing their growth rates and rendering them more susceptible to mortality (Tops et al., 2009). Once mature, the infective spores are released into the surrounding water column continuing the lifecycle.

The susceptibility to *T. bryosalmonae* differs between salmonid species (Syrová et al., 2020). *T. bryosalmonae* infects salmonids through spores, mainly via the gills, and spreads via the bloodstream to infect organs, mainly the kidneys (Grabner & El-Matbouli, 2010; Morris et al., 2000). The parasite enters its proliferation stage after 20-30 days post exposure (Bettge et al., 2009), where the shedding of DNA is highest after 55 dpe around 15°C (Strepparava et al.,

2018). Once matured, the unique parasite daughter spore can be transmitted from the salmonid fish to bryozoans through urine (Grabner & El-Matbouli, 2008; Morris & Adams, 2006). The presence of sporogonic parasite spores inside the salmonid hosts suggest *T. bryosalmonae* being active in their lifecycle. The species in where such spores are observed include the Atlantic salmon *Salmo salar,* brown trout *Salmo trutta,* brook trout *Salvelinus fontinalis,* Artic charr *Salvelinus alpinus*. (Abd-Elfattah et al., 2014; Grabner & El-Matbouli, 2008; Svavarsdóttir et al., 2021). Whereas rainbow trout *Oncorhynchus mykiss* and grayling *Thymallus thymallus* are among species not being capable of producing spores that can infect bryozoans (Abd-Elfattah et al., 2014; Grabner & El-Matbouli, 2008).

In the coming years, future projections in Norway indicate the mean annual air temperature will increase by 2.7 °C for scenario RCP 4.5 and 4.5 °C for scenario RCP 8.5 until the end of the century (Hanssen-Bauer et al., 2009). The correlation between air temperature and water temperature is complex, where an average 1 °C increase in air temperature corresponds to 0.6 – 0.8 °C increase in the water temperature worldwide (Morrill et al., 2005).

The salmonid fishes are prone to developing PKD when the water temperature reaches the ranges of 12-18 °C for a prolonged period (Clifton-Hadley et al., 1986). For brown trout and rainbow trout studies show that the highest mortality and morbidity occur when the temperature are over 15°C (Bettge et al., 2009; Strepparava et al., 2018; Waldner et al., 2021). In River Åelva considerable mortality occurred in 0+ atlantic salmon, where autopsy revealed as diagnosis to be result of PKD and high temperatures (Sterud et al., 2007).

The high-water temperatures will accelerate proliferation rate inside the kidney (Tops et al., 2006). As the parasite persists in disrupting the normal functioning of the kidney, the fish displays inflammatory reactions, including kidney hyperplasia, kidney enlargement, spleen swelling, and anaemia (Bruneaux et al., 2017; Sudhagar et al., 2020). Recovery from PKD is dependent on the temperature and the immune responses of the salmonid fish (Bailey

et al., 2017). In some instances, the high-water temperatures can speed up the recovery time and salmonid fish can fully clear the infection from their kidneys, but can result in higher mortality (Waldner et al., 2021).

The salmonid and bryozoan host may be infected with *T. bryosalmonae* throughout the year, but the severity of the disease in salmonids will not develop unless the favourable conditions are present (Waldner et al., 2021). Environmental stressors and climate change may increase susceptibility for diseases and *T. bryosalmonae*, consequently diminishing their health and populations. (Mustafa et al., 2024; Waldner et al., 2021)

This study is a part of the project, "Påvirker nyresykdommen PKD overlevelsen til sjøørretunger og -smolt i Sandvikselva" with The Norwegian Institute for Nature Research (NINA). This thesis aims to determine the presence of the myxozoan parasite *Tetracapsuloides bryosalmonae* at 5 different stations within River Sandvikselva by eDNA analysis during summer season.

Methods

Study area

River Sandvikselva is a part of a water system that stretches north from Hole and Ringerike through Bærum municipality and has its outlet into the Oslofjord (Figure 2). River Sandvikselva is 6.2 km long (Miljødirektoratet, 2024), and has a catchment area of 193.39 km^2 (NVE, 2024). Vann-nett has given the River Sandvikelva water body ID 008-94-R and classifies it as a water-type R110 (Miljødirektoratet, 2024).

During July 2023, a climatological overview of the precipitation of Norway by Meterological Institute shows on average 25 % more precipitation than the normal period (Meterologisk Institutt, 2023a). Additionally, River Sandvikselva was affected by high water flow and flooding due to the extreme weather Hans in August (Meteorologisk Institutt, 2023b).

Five water sampling locations were chosen from advice of Tor Atle Mo at NINA and can be found in Table 1. Station 1 was closest to the river outlet, station 5 highest up in the watercourse. Due to road constructions in the weeks 31-39, station 4 and 5 became unavailable. Station 4 b downstream the construction site had to be added, totalling 6 stations in this survey.

Table 1

Sampling locations with UTM coordinates

Sampling locations	UTM zone 32N, E	
Station 1	6640883.39	583778.26
Station 2	6641994.04	582888.82
Station 3	6644764.78	580301.01
Station 4	6645355.82	579811.12
Station 4 b	6645174.66	579911.84
Station 5	6645644.55	579647.88

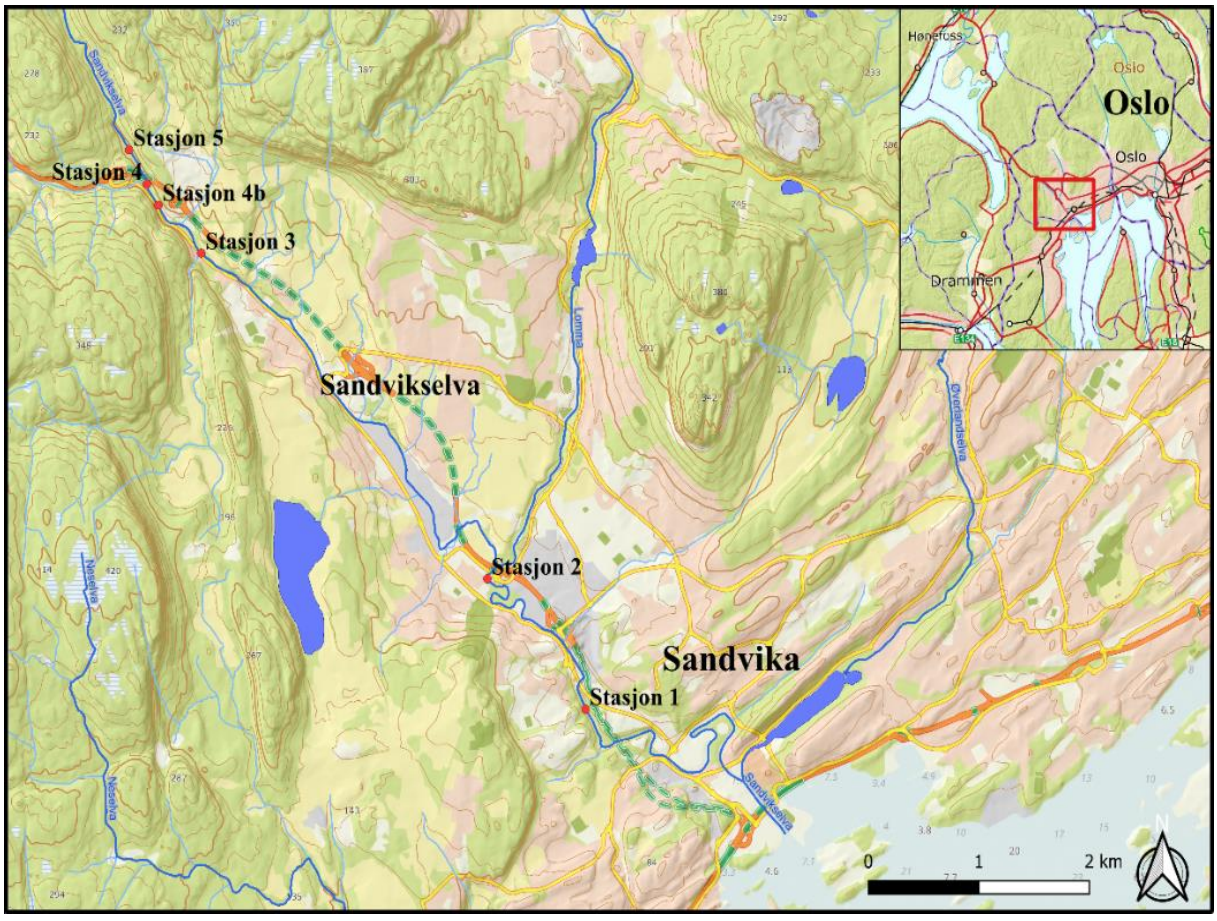

Figure 2. Map of the study area. WMS accessible on Geonorge.no was used to create this map, coordinate reference system used: ETR89/UTM zone 32N, EPSG:25832.

In the surroundings of the stations several different types of anthropogenic activity pose significant influence on River Sandvikselva. Among those are the urban area of Sandvika, the expansion of roads, the modifying of watercourses and various activities that results in pollution. Moreover, as depicted in Bøhler (2017), there are many pipes with potential polluting water draining into the river. Additionally, many point sources of pollution are registered on vann-nett database (Miljødirektoratet, 2024), shown in Figure 3.

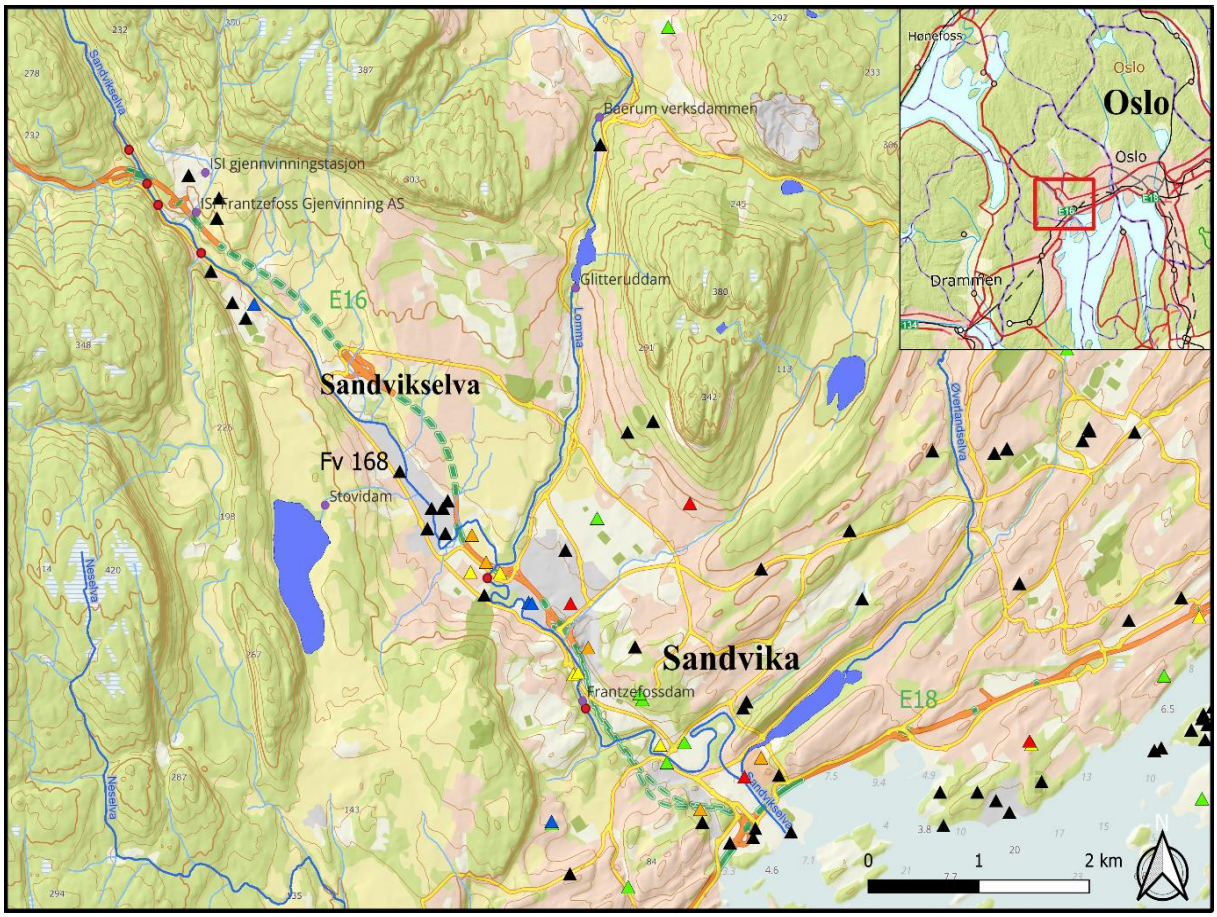

Figure 3. Overview of significant anthropogenic activity in Sandvika. Triangles in the map are point source pollutions, green=low/not polluted, yellow= acceptable pollution, red= unacceptable pollution and need for action, black=suspicion of pollution without survey. WMS accessible on Geonorge.no was used to create this map, coordinate reference system used: ETR89/UTM zone 32N, EPSG:25832.

Field work

Water sampling

Water samples were taken from June to September. To avoid stirring up mud in the river, the 10 L water container was filled by entering the river downstream of the sampling area and water was collected upstream from the standing position. The water temperature was measured using a thermometer. List of the sampling equipment can be found in attachment (Table A1).

Latex gloves were used to reduce cross-contamination during sampling and was changed between every water sample (Sahu et al., 2023).

Sampling was done following a modified protocol for the use of Peristaltic pumps in aquatic environment from Nature Metrics (Nature Metrics, 2024). The filtration system was set up by attaching the silicone hose to the "inlet" side of the Nature Metrics filter (GF 5.0 µm/PES 0.8 µm) and placing the other end of the hose in the water container. The filter is made up by two layers, one with glass-fiber filter with the pore size of 5.0 µm which can filter out the larger particles, and a Polyethersulfone filter with a pore size of 0.8 µm that collects the smaller particles.

By connecting the hose to a Vampire Sampler (pump) and turning it on, the filtration process started. 10-liters of water were supposed to be filtered, but sometimes the filter became cloggeddue to the particle content of the water, and less volume was obtained. For conservation of the filtered material 3 mL ATL buffer was applied to the filter. The filters were stored inside a marked ziplock bag placed inside a backpack in the shade until all sampling were completed. When sampling was done for the day, the marked zip-lock bags were stored in the dark, at room temperature at USN.

Safety measures included the use of waders and life jackets, with an additional person observing from the shore.

Figure 4. Sampling equipment

Cleaning protocol: Water sampling

Before and after fieldwork the following cleaning protocol was used. Firstly, the silicone hoses were placed in the water containers, which were subsequently filled with 1 liter of water, vigorously shaken for 1 minute, and then drained. Following this, the containers were refilled with a solution of equal parts chlorine and water, shaken for another minute, and emptied. This process was repeated twice to ensure thorough cleaning. In the end, the silicone hoses and containers underwent a thoroughly rinsing with distilled water to complete the procedure. The 10-Liter water container and silicone tubing at each site were washed between sampling dates.

Molecular analysis

DNA-extraction

For extraction of eDNA from the metrics filters sampled in River Sandvikselva, NINAs revised protocol for isolation of DNA from water-filters for eDNA analysis was used (Brandsegg, 2023). A visualization of the first steps can be seen in Figure 5 and the list equipment used can be found in Table A2. The process started by separating the plunger of a BD Plastipak 50 mL syringe from its barrel. Thereafter, the barrel was connected into the filter on the "inlet" side and a pipettor was used to add 130 µl 10% Proteinase K (2 mg/ml) into the opening. The plunger was connected to the barrel and was pressed slowly and controlled down to add proteinase K onto the filter. When proteinase K was successfully applied, the syringe was placed back into its branded case for later use. The red caps were turned back on, and the filters were placed in a heating cabinet overnight at 56 °C.

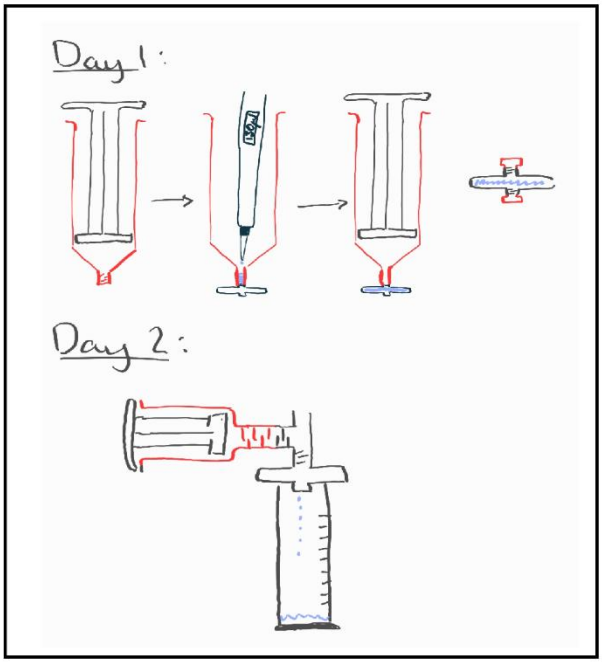

Figure 5. DNA extraction: Simple visualisation of starting steps

On the next day, the modified protocol for DNA extraction of Qiagens Blood and Tissue-kit was used (Brandsegg, 2023). The red filter-caps were removed, and each filter was placed on the top of a 15.0 mL tube with the “inlet” side of the filter facing down. A Luer coupler was then connected to the “outlet” side of the filter. The syringe was connected to the coupler and was used to press air into the filter for filter-content to come out. The process stopped when only foam came out of the filter. This was suggested from the supervisor and Prof. Jenkins due to risk of aerosols busting, that could lead to contamination of the air and samples. 1000 µL AL-buffer and 1000 µL ice-cold 96 % ethanol was added to the sample lysate and vortexed before the next step.

The supernatant was loaded into the Spin Column in several steps due to the large volume, using a pipettor, and thereafter centrifuged at 14800 rpm for 2 minutes. After centrifugation, the eluate was discarded, this process continued until all sample material had been centrifuged.

Afterwards, the Spin Column containing the sample material was washed with 1000 µL AW1 buffer and centrifugated at 14800 rpm for 2 minutes. Due to the Spin Column not being able to contain 1000 µL AW1 buffer, the sample material underwent washing and centrifugation two times with 500 µL each time, totalling 1000 µL. As before, the supernatant from the Spin Column was discarded after each centrifugation.

Following that, the Spin Column was washed with 3000 µL AW2 buffer and centrifuged at 14800 rpm for 10 minutes. Due to the sample problem, the sample material underwent washing and centrifugation six times with 600 µL each time, totalling 1000 µL. As before, the supernatant from the Spin Column was discarded after each centrifugation.

The sample material was transferred to a collection-tube, and it was used a pipettor to add 200 µL of preheated AE buffer (56 °C) to the Spin Column. Before the next step a 10-minute incubation period needed to pass before centrifuging on 14800 rpm for 2 minutes. After

centrifugation, the eluate was transferred back to the spin column. Another, incubate period for 10 minutes needed to pass before centrifugation on 14800 rpm for 2 minutes. AE buffer was used to release the purified DNA from the membrane and ensures concentrated DNA can be used in the downstream processes.

Lastly the DNA material was transferred to a 1.5 mL lo-bind Eppendorf tubes, the DNA content was measured by NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific), before storage in a refrigerator.

Cleaning protocol: DNA extraction

During the DNA extraction, the Luer Coupler had to be washed between each sample doing the following. Firstly, setting up the washing station by mixing solutions of equal parts chlorine and water in two separate beakers marking one for soaking and the other for cleaning. A third beaker was filled with distilled water for rinsing. A BD Plastipak 50 mL syringe designated for cleaning was placed in the cleaning-beaker and another BD Plastipak syringe was placed in the rinsing-beaker.

Starting the cleaning protocol by removing the coupler from the soaking-beaker with a tweezer. The cleaning-syringe was then used to inject the chlorine-water solution into the coupler washing the insides. Thereafter, the cleaning-syringe was disconnected and placed back into the cleaning-beaker. Afterwards, the rinsing-syringe with distilled water was connected to the coupler rinsing the insides of the coupler. Lastly the rinsing-syringe was used to rinse the tweezers and the outside of the coupler. The clean Luer Coupler and tweezers was placed on a clean tissue of paper for drying and for later use.

qPCR

To detect for *T. bryosalmonae* 18S rDNA was used, the reagents and end concentrations can be found in Table 2.

The forward primer used was PKDtaqf1 (5' GCGAGATTTGTTGCATTTAAAAAG-3') (Bettge et al., 2009), the reverse primer PKDtaqr1 (5'-GCACATGCAGTGTCCAATCG-3') (Bettge et al., 2009), and the probe VIC-dye TaqMan MGB (5'-AGTCGGACGGTTCCA-3') (Andrew Jenkins, USN).

Table 2:

Reagents and end concentrations per sample

Reagents	Volume pr sample µL	End concentration in 25 µL
Mastermix 2x	12.5	
Forward primer	1	400 nM
Reverse primer	1	400 nM
Probe	0.5	200 nM
Water	0.5	
Sample	5	
Total volume	25	

From the River Sandvikselva 44 water samples were collected in total. The qPCR-analyses included three technical replicates of each sample (n = 132), three blank controls (filtered destilled water), two negative controls (sterile water) and three positive controls (samples from brown trout kidney positive for *T. bryosalmonae* "POSFYN49"). Additionally, each qPCR-run included a dilution series ($1:10^1$, $1:10^2$, $1:10^3$ $1:10^4$, $1:10^5$ and $1:10^6$) of known concentrations from a synthetic plasmid, pTbr, which is the cloning vector pUC57 with an insertion of the *T. bryosalmonae* target sequence (Oredalen et al., 2022). The known concentrations can be found in Table 3, 1 copy = 1 plasmid with the target nucleotid sequence of 76 basepairs (bp).

The qPCR program was as follows: one cycle for 10 minutes at 95 °C, followed by 45 cycles for 15 seconds each at 95 °C, then 45 cycles for 1 minute each at 60 °C. Lastly 10 minutes cycle at 4 °C.

Table 3:

Dilution series: Plasmid pTBR

Dilution		Copies/µL	5 µL plasmid in 25 µL well	Copies in 25 µL
1:10	(10 µL usersolution + 90 µL TE-buffer)	200000	40000	1000000
$1:10^2$	(10 µL solution of 1:10 + 90 µL TE-buffer)	20000	4000	100000
$1:10^3$	(10 µL solution of $1:10^2$ + 90 µL TE-buffer)	2000	400	10000
$1:10^4$	(10 µL solution of $1:10^3$ + 90 µL TE-buffer)	200	40	1000
$1:10^5$	(10 µL solution of $1:10^4$ + 90 µL TE-buffer)	20	4	100
$1:10^6$	(10 µL solution of $1:10^5$ + 90 µL TE-buffer)	2	0,4	10

Note: 1 copy = 1 plasmid with insertion of one nucleotid sequence of 76 basepairs (bp)

The Ct value in the qPCR run is where the PCR curve intersects with the linear rising part of the curve (GenomicPlatform, 2024). The higher the Ct, the more cycles of amplifications it is needed to detect fluorescence (GenomicPlatform, 2024), and the lower initial concentration of the target nucleotide is present in the sample. Analysing the results, a positive sample is defined as one where at least two of three PCR replicates shows positive Ct values, and where the amplification curves are parallel with the standard curves (Figure 6).

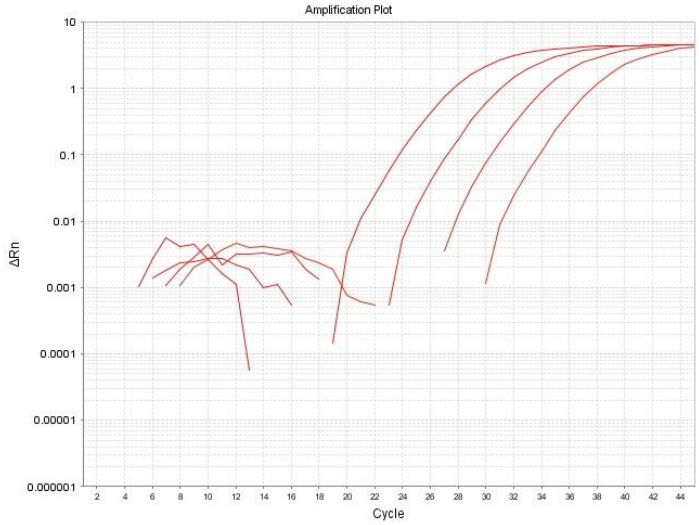

Figure 6. Dilution series: Amplification plot. Standard curve concentrations ranging from: $1{:}10^{1}$ (40000 copies), $1{:}10^{2}$ (4000 copies), $1{:}10^{3}$ (400 copies) and $1{:}10^{4}$ (40 copies), $1{:}10^{5}$ (4 copies) and $1{:}10^{6}$ (0.4 copies) did not amplify.

Statistical analysis

The qPCR analyses were extracted into excel by StepOne^{TM} Software v 2.3. The samples with their triplicates were sorted to their respective stations where the means and medians were calculated to observe distribution or extreme values. Additionally, a boxplot was created in Rstudio [64-bit] R-4.3.0, to better visualize potential outliers.

From the software, the amplification plots and standard curves were also exported (figure 6 – figure 10).

Results

All qPCR results are shown in Appendix Table A3. Positive samples of *T. bryosalmonae* were found in two of six stations (Table 4). In station 1 it was detected in 11 replicates and in 5 replicates at station 2, one of them being in September. Across both stations, there were 4 positive samples at station 1 and 1 positive sample at station 2.

The mean Ct values for the technical replicates of the samples taken each date were calculated (Table 5). For station 1, the means are 33.48 (July) and 33.78 (August). For station 2, the means are 34.10 (July) and 36.25 (September).

The median Ct values were calculated based on the same procedure as for the mean values (Table 6). For station 1, the medians are 32.67 (July) and 33.16 (August). For station 2, the medians are 34.16 (July) and 36.25 (September).

Water temperature measurements for the six stations from June to October can be found in Table 7. The highest temperature was measured in Juli (17°C - 15°C) and the lowest temperature was 3 – 4 °C in October.

The amplification plots can be seen in Figure 8 and Figure 9. In these plots, standard concentration $1{:}10^5$ and $1{:}10^6$ did not amplify. The results from the standard curve plot in Figure 10, show that most of the-positive samples from River Sandvikselva appearing at CT values higher than plasmid dilution of $1{:}10^{4,}$ equivalent to concentrations lower than approximate 40 copies of target nucleotide in the 25 µL sample analysed.

Table 4

Positive replicates of Tetracapsuloides bryosalmonae in River Sandvikselva

Sample ID	Month	Station	Ct1	Ct2	Ct3
11	July	Station 1	31.77	32.67	31.88
12	July	Station 1	35.49	35.60	Undetermined
13	July	Station 2	Undetermined	33.99	Undetermined
14	July	Station 2	34.33	34.80	33.29
21	August	Station 1	32.47	33.52	32.78
22	August	Station 1	33.13	33.20	33.78
31	September	Station 2	Undetermined	36.25	Undetermined

Note: Positive samples are shown with Ct values, representing number of cycles required for the detection of the parasite (target nucleotide) in each sample. Undetermined results show inconclusive results.

Table 5

Mean Ct values: qPCR analysis of Tetracapsuloides bryosalmonae in River Sandvikselva

Month	Station 1	Station 2	Station 3	Station 4	Station 4b	Station 5
June	Undetermined	Undetermined	Undetermined	Undetermined	*	Undetermined
July	33.48	34.10	Undetermined	Undetermined	*	Undetermined
August	33.78	Undetermined	Undetermined	*	Undetermined	*
Sept	Undetermined	36.25	Undetermined	*	Undetermined	*
Oct	Undetermined	Undetermined	Undetermined	*	Undetermined	*

Note: * gaps in the sampling period.

Table 6

Median Ct values: qPCR analysis of Tetracapsuloides bryosalmonae in River Sandvikselva

Month	Station 1	Station 2	Station 3	Station 4	Station 4b	Station 5
June	Undetermined	Undetermined	Undetermined	Undetermined	*	Undetermined
July	32.67	34.16	Undetermined	Undetermined	*	Undetermined
August	33.16	Undetermined	Undetermined	*	Undetermined	*
Sept	Undetermined	36.25	Undetermined	*	Undetermined	*
Oct	Undetermined	Undetermined	Undetermined	*	Undetermined	*

Note: * gaps in the sampling period.

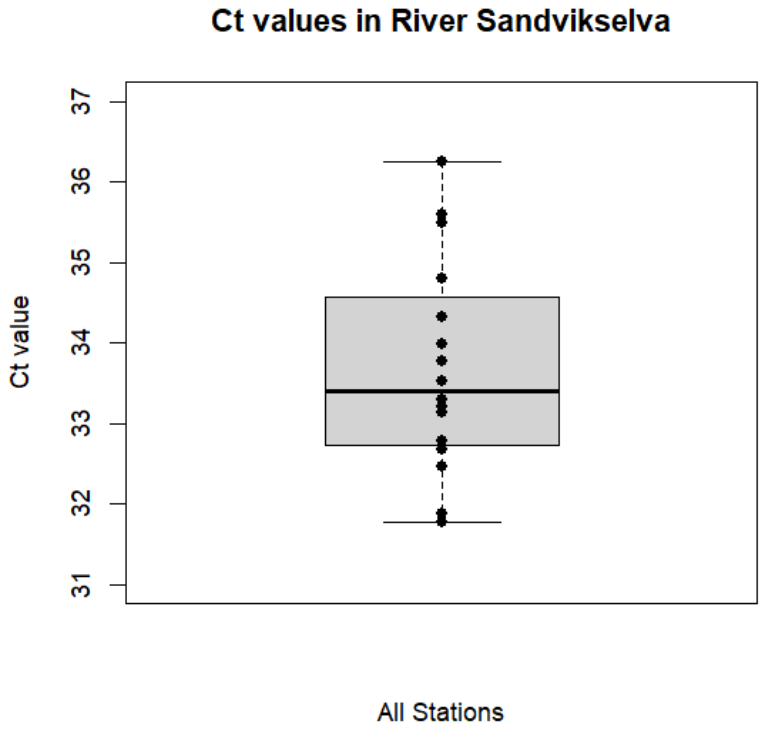

Figure 7: Boxplot of C_T values in River Sandvikselva

Table 7

Water temperature measured in °C River *Sandvikelva*

Month	Station 1	Station 2	Station 3	Station 4	Station 4b	Station 5
June	13.7	13.7	13.7	13.7	*	13.7
July	17.0	17.0	15.0	15.0	*	15.0
August	14.0	14.5	10.0	*	10.0	*
September	10.0	10.5	10.0	*	8.0	*
October	4.0	3.0	3.0	*	3.5	*

Note: * gaps in the sampling period.

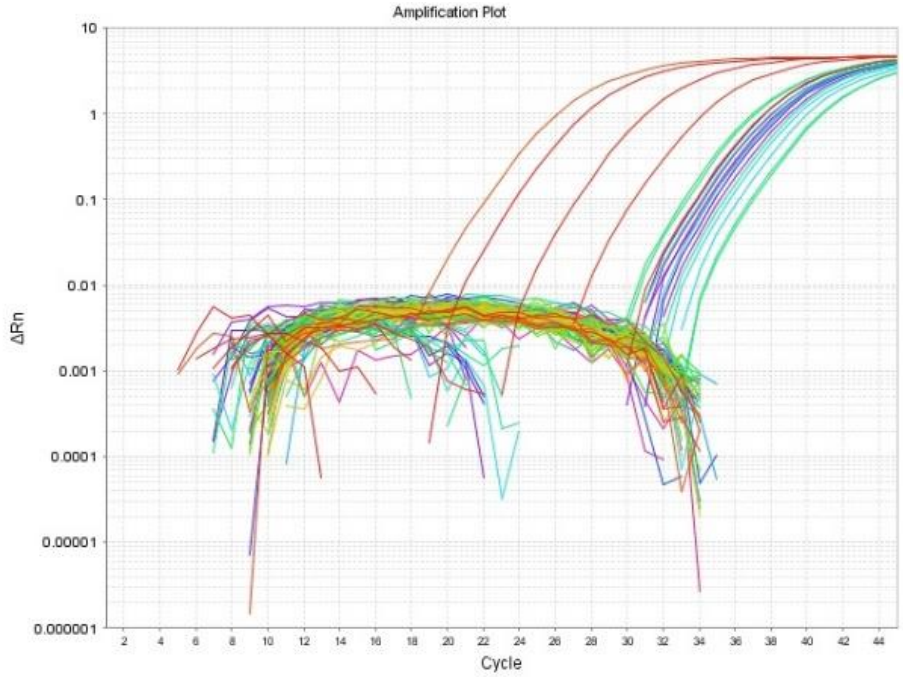

Figure 8. qPCR Amplification: June, Juli, and August.

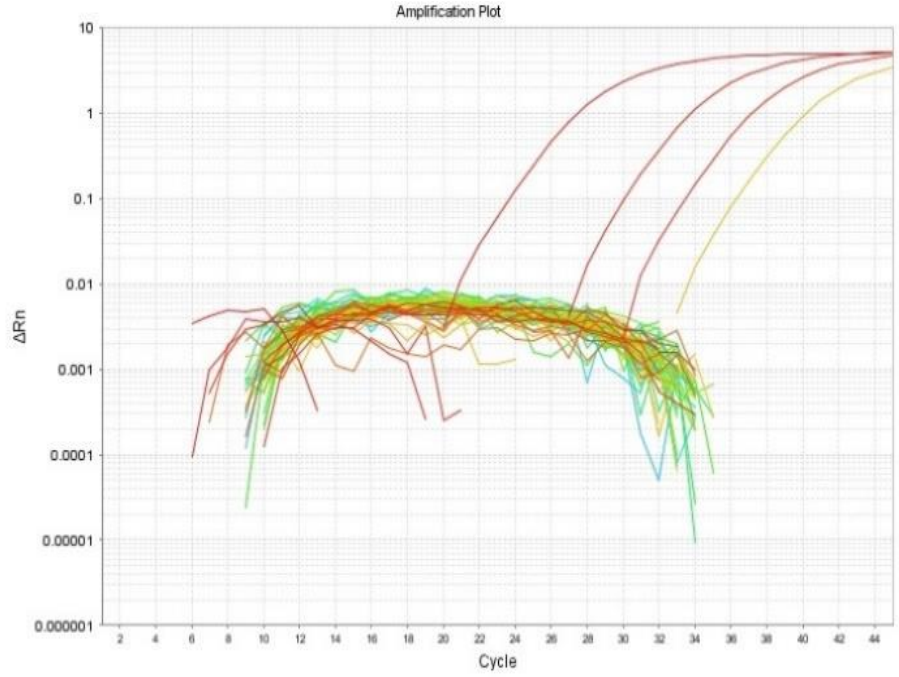

Figure 9. Amplification plot: September & November.

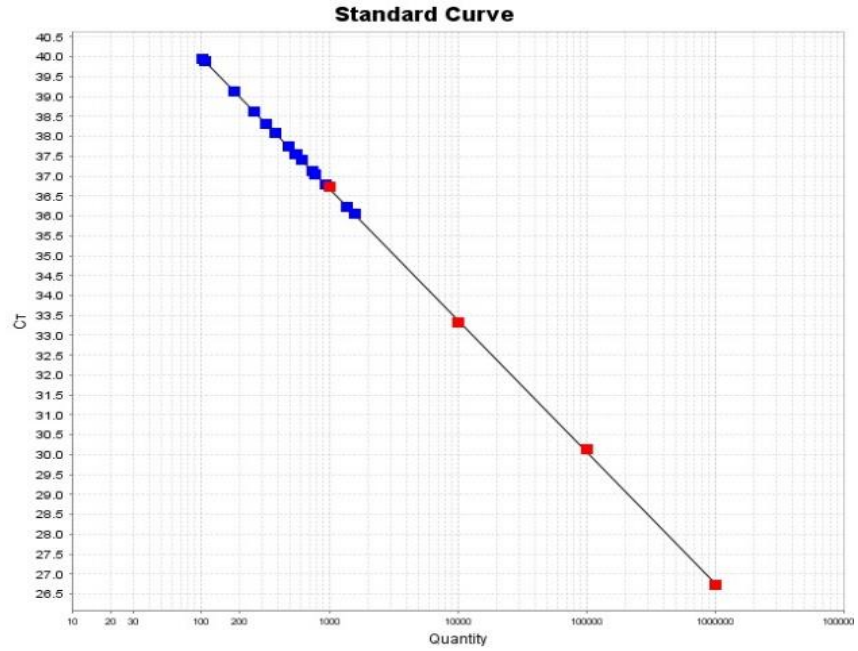

Figure 10. Standard curve: June, Juli & August

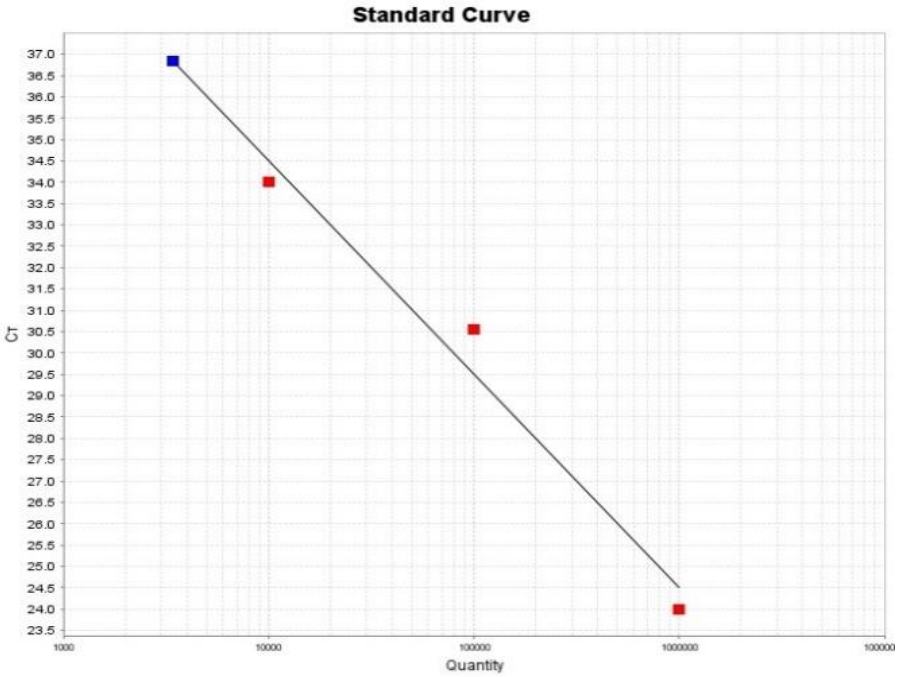

Figure 11. Standard curve: September & October

Discussion

Positive samples of *T. bryosalmonae* were found at station 1 and station 2 in River Sandvikselva (Table 4).

The mean (Table 5) and median (Table 6) Ct values for station 1 in July were 33.48 (mean) and 32.67 (median), and at station 2 they were 34.10 (mean) and 34.16 (median). In August, the values at station 1 were 33.78 (mean) and 33.16 (median). The Ct mean and Ct median values and the boxplot from Figure 7 show consistency in the distribution of the results and no biases or extreme values.

The highest presence of samples meeting the criteria for a positive sample was at station 1, where 4 out of 4 field samples were positive during Juli and August, while at station 2 there was only 1 positive sample in July (Table 4). All positive samples were detected in July and August, when the temperature was at its highest with 17 °C and 14 °C respectively. This coincides with the temperature ranges where the parasite proliferates the most in its hosts, leading to a higher prevalence of spores in the water, which is in line with findings done by others (Clifton-Hadley et al., 1986; Strepparava et al., 2018; Tops et al., 2006). Based on these findings it is concluded that the myxozoan parasite *T. bryosalmonae* is present in River Sandvikselva using eDNA analysis.

Samples from stations 1 and 2 during June was negative regarding parasite DNA. At this time the water temperature was 13.7 °C and is in the lower range of temperature for parasite presence. The results from June are comparable with a former study in River Sandvikselva (Skrutvold & Roseth, 2023). The study strengthens the results as it uses similar water sampling method including the same Vampire Sampler and Nature Metrics filters. However, they did not extend the sampling to include more locations and did not extend the sampling period over time. The unexpected result in June can be due to that the proliferation phase or the highest

intensity of proliferation has not yet been reached, as explained by others (Bettge et al., 2009; Strepparava et al., 2018). The “lag” of spore production most probably will limit the amount of parasite spores from salmonids in the water in June.

However, according to Carraro (2018), bryozoans release disproportionately more spores than fish hosts. River Sandvikselva is nearby urban areas depicted in Figure 3 where pollution is likely to come from sewage run-off resulting in eutrophication and abundance of phosphorus and nitrogen (Strokal et al., 2021). Based on Hartikainen (2009) bryozoans responds to increased nutrient concentrations by increased growth resulting in higher biomass (Hartikainen et al., 2009). Therefore, it should be more abundance of spores from bryozoans and the expected reduced production of salmonid spores should possibly not be a limiting factor in detecting *T. bryosalmonae* in River Sandvikselva.

Detection errors due to challenges during sampling and analysis pose a challenge in eDNA analyses and can potentially affect the reliability of *T. bryosalmonae* detection. One abnormal replicate was detected at station 2 in September (Table 4 with a C_T value of 36.25. The water temperature at station 2 during sampling in September was 10.5 °C (Table 7). The abnormal replicate was treated as a true detection, but not positive sample due to the criteria set. However, the detection in only one replicate may be the result of low concentration of parasite spores in the river, or contamination in the field or in the lab. Since this is the only positive sample during the sampling month, and were stored separately from other positive samples, it is unlikely that contamination has happened. Another possibility is during DNA extraction and qPCR. To reduce the detection errors and contamination risks, supervisor and Professor Jenkins demonstrated how to use the protocols correctly, ensured close supervision and were available for the next protocol steps. Low concentration of parasite spores seems to be the most likely reason for the low detection rate, as water temperature in the river was 10.5

degrees Celsius (Table 7) below the temperature where the production of spores is high (Clifton-Hadley et al., 1986; Strepparava et al., 2018; Tops et al., 2006).

Furthermore, the negative samples at stations 3, 4, 4b and 5 may indicate that *T. bryosalmonae* has not been established further upstream of station 2. This could mean that the parasite is absent at these stations. However, it could also mean that the concentration of parasite spores is too low to be determined, resulting in false negatives.

The standard curve in the PCR analysis revealed relatively low sensitivity, with only detection of *T. bryosalmonae* at plasmid concentration below $1{:}10^4$ (Figure 10). Therefore, false negative results are more likely to occur outside the months that are favourable for *T. bryosalmonae* due to lower spore concentrations in the water column.

According to Wood, eDNA from fish is highly concentrated but heterogeneous. The heterogeneity of the eDNA makes it unevenly distributed and reduces the detection rate of *T. bryosalmonae* spores (Wood et al., 2021). Furthermore, the effects of precipitation during Juli (Meterologisk-Institutt, 2023a) and the extreme weather Hans (Meteorologisk-Institutt, 2023b), may have led to increased water flow, which could have diluted the DNA concentrations. This dilution might have contributed to the difficulty of detecting *T. bryosalmonae* in the survey, as observed by others (Wood et al., 2021).

Considering the heterogeneity, water flow and as well as analysis cost, it was decided to use 2 replicates (instead of 3 replicates) of 10 L water sampling volume and closed filters in the field to optimise the detection rate (Rees et al., 2014; Spens et al., 2017). eDNA can be transported over long distances and it is likely to accumulate downstream in stations (Jane et al., 2015), such as stations 1 and 2, and not stations 3, 4, 4b and 5.

The results highlight the relatively low sensitivity and detection rate below $1{:}10^4$, as illustrated in the standard curve plot in Figure 10. The higher dilutions $1{:}10^5$ and $1{:}10^6$ failed to amplify in both amplification processes, seen in Figure 8 and Figure 9. To accurately

pinpoint the ranges of the concentrations below $1:10^4$ has been reduced. However, most concentrations of the replicates fall below $1:10^4$, and it is for certain less than 40 copies of the nucleotid sequence (Table 3).

This underscores the detectability of *T. bryosalmonae* within the concentrations limiting detection of true positive samples. The inability to amplify at higher dilutions suggests the detection threshold of eDNA analysis for *T. bryosalmonae*, highlighting the need for refined methodologies or alternative approaches to enhance sensitivity and accuracy in low-concentration environments such in the upstream stations.

Traditional survey methods vs eDNA analysis methods

Traditional methods often involve direct observations and eDNA methods involve indirect observations sampling from the environment. It is difficult to observe *T. bryosalmonae* in the environment and it is therefore needed to investigate their hosts. In the cases of salmonid fish, it is possible to observe through outbreaks of symptoms, even when fish have high parasite concentration in the liver without visible symptoms. Since salmonid fish can be infected with *T. bryosalmonae* throughout the year, fish can be collected by electrofishing. This has been done in River Sandvikselva before by Mo & Jørgensen (2017). Another traditional method is by collecting bryozoans (Bendixby & Hals, 2009). However, through the traditional methods the surveyer is required to kill the salmonid or bryozoan host before examining. The invasive method can result in decline in the vulnerable populations you are trying to preserve. In comparison, eDNA is a non-invasive method, where it is not needed to euthanize salmonid fish or bryozoans since it is possible to sample DNA from the environment (Taberlet et al., 2012).

eDNA is connected with detection errors mentioned and additionally has several limitations due to lack of standardizations of methods and protocol for surveys (Fediajevaite et al., 2021). For example, currently there are no guidance regarding requirements of positive samples. Studies such as Sieber (2023) and Stelzer (2024) states that one positive sample is

sufficient if the parasite is present in at least one of the three replicates. For Ines-Fontes (2017a) a positive sample is if two of two replicates are positive or such as Skrutvold & Roseth (2023) implementing strict criteria by having at least two of three replicates amplifying.

Additionally, training and certification is needed to conduct electrofishing sampling effectively and safely. Meanwhile eDNA sampling protocols are relatively simple and how to mitigate risk and contamination is often highlighted. Electrofishing equipment is not readily available to the public, whereas Nature Metrics kits are readily available for use for everyone in every country (Nature-Metrics, 2024). The average enthusiastic person has the means to do sampling for PCR analyses without much training. However, with expensive lab equipment and limited training, results could become less trustworthy. This step is mitigated by Nature Metric by being able to return the sampling kits back to their laboratory for analysis (Nature Metrics, 2024). The possibility of an enthusiastic person to do sampling for eDNA analysis, where Nature Metrics place the findings in a database so experts in the field can conduct their analysis and verify findings. The collection of eDNA can be gathered in a database that involves the public in scientific research, called Citizen science (European Commission, 2024). It could drastically increase accessibility to interesting results about species distribution based on eDNA, since traditional sampling is often time consuming, labour intensive and expensive when identifying species that are elusive (Beng & Corlett, 2020).

When it comes to the sensitivity of analysis, eDNA sampling is considered more sensitive than traditional sampling, being able to detect a wide range of species especially in aquatic environments for biomonitoring (Rishan et al., 2023). The detection of fish parasites traditionally has been dependent on gathering fish and then euthanizing them which is not required for with eDNA surveys.

However, as discussed earlier about sensitivity and the difficulty in this survey to detect outside favourable conditions for *T. bryosalmonae,* it should be considered to implement

traditional sampling by either salmonid or bryozoan host along with eDNA sampling to make sure false negative detections are limited. By combining both traditional sampling with eDNA it is possible to achieve more comprehensive and reliable assessment of presence of *T. bryosalmonae.*

Sources of error

Potential sources of error in this survey include variables such as water flow, temperature, and inhibitions. Water flow measurements was not considered when sampling started, which is an important factor in the dilution and distribution of DNA discussed earlier. Water flow in River Sandvikselva fluctuated in months of Juli and August (Meteorologisk-Institutt, 2023b; Meterologisk-Institutt, 2023a). When sampling during July and August were made during the high-water levels, the water flow was not recorded. Additionally, water temperature is another variable that was not measured continuously. Water temperature fluctuates throughout the day depending on when it's being measured and throughout the month. More measurements should've been taken to observe if there were periods where the temperature was abnormally high that could've contributed to parasite spore production. Lastly, testing for inhibitions could have been done by spiking samples with the positive control, but the amplification curves did not indicate any inhibition.

A limiting factor in this survey was the funding and the budget received for the master-thesis. With unlimited funding it could have been considered to take samples every two weeks instead of monthly and three replicates in the field instead of two. This however would be time consuming and costly due to the long distance from Bø to River Sandvikselva being almost a two-hour drive. Additionally, without a car and available assistance it would be difficult to do sampling more frequently. Furthermore, if the lack of resources would not be an issue it could've been considered to do another qPCR round for the dilution series, due to highest dilutions not amplifying.

Conclusion

The emerging myxozoan parasite *T. bryosalmonae* is causative to Proliferative kidney disease in salmonid fishes. Detecting the presence of *T. bryosalmonae* by eDNA analysis in River Sandvikselva successfully is important for the conservation and ecological management of salmonid species. Climate change and future temperature predictions may worsen the living conditions of salmonids by exacerbating symptoms leading to higher morbidity. Environmental DNA has some drawbacks and shortcomings, but by using optimized methods together with traditional methods it is possible to minimize errors and more accurately detect elusive species in rivers. In this way, it is possible to monitor larger areas of interest more frequently and cost-effectively.

Moving forward, it is important to establish standardized methods and protocols among experts, making it easier to reproduce and compare surveys, and to make informed decisions about the conservation of bryozoans and salmonids facing threats such as Proliferative kidney disease.

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Appendix A

Table A1

Water sampling equipment

Equipment:		
10 L water container	5	Biltema vanndunk 10 L
Silicone hose	5 meters	
Filters	46	Sterlitech, GF 5.0 µL/PES 0.8 µm, Female Luer Lock Inlet, Male Luer Lock Outlet
ATL buffer	138 mL	
Plastic syringe, 3 mL, luer lock	46	
Combi stopper	46	
Filtering system		Bürkle Vampire Probensammler
Thermometer		
Strips		Biltema strips
Ducttape		Biltema gaffateip sort
Gloves		VWR® Powder-Free Latex Examination Gloves
Water wader		
Lifejacket		

Table A2

DNA extraction equipment

Equipment		Name
1-100 µL pipettor	1	
20-200 µL pipettor	1	
100-1000 µL pipettor	1	
10 µL pipette tips		
200 µL pipette tips		
1000 µL pipette tips		
Syringe 50 mL	46	BD Plastipak 50 mL syringe
Valves	2	Luer Coupler
15.0 mL tubes	46	
Microcentrifuge 2.0 mL tubes	46	Cole-Parmer, Microcentrifuge Tubes Click Close
Spin column	46	
1.5 mL tubes	46	Eppendorf Lo-bind Tubes
AL-buffer	46 mL	Buffer AL Lysis buffer
AW1-buffer	46 mL	QIAGEN, Buffer AW1 Wash buffer 1
AW2-buffer	138 mL	QIAGEN, Buffer AW2 Wash buffer 2
96 % ethanol	46 mL	
10 % Proteinase K (2 mg/ml)	6 mL	
Mini vortex mixer		
Heating cabinet		Termaks heating cabinet, 0-60 °C
Centrifuge		Thermo SCIENTIFIC, MicroCL 21 centrifuge

Table A3

qPCR Analysis of Tetracapsuloides bryosalmonae in the River Sandvikelva

Sample ID	Date	Location	Ct Replicate A	Ct Replicate B	Ct Replicate C	Quantity replicate A	Quantity replicate B	Quantity replicate C
1	05/06/2023	Station 1	Undetermined	Undetermined	Undetermined			
2	05/06/2023	Station 1	Undetermined	Undetermined	Undetermined			
3	05/06/2023	Station 2	Undetermined	Undetermined	Undetermined			
4	05/06/2023	Station 2	Undetermined	Undetermined	Undetermined			
5	05/06/2023	Station 3	Undetermined	Undetermined	Undetermined			
6	05/06/2023	Station 3	Undetermined	Undetermined	Undetermined			
7	05/06/2023	Station 4	Undetermined	Undetermined	Undetermined			
8	05/06/2023	Station 4	Undetermined	Undetermined	Undetermined			
9	05/06/2023	Station 5	Undetermined	Undetermined	Undetermined			
10	05/06/2023	Station 5	Undetermined	Undetermined	Undetermined			
11	14/07/2023	Station 1	31.77	32.67	31.88	1533.4	822.52	1421.77
12	14/07/2023	Station 1	35.49	35.6	Undetermined	118.64	109.96	
13	14/07/2023	Station 2	Undetermined	33.99	Undetermined		331.55	
14	14/07/2023	Station 2	34.33	34.8	33.29	263.37	190.03	539.03
15	14/07/2023	Station 3	Undetermined	Undetermined	Undetermined			
16	14/07/2023	Station 3	Undetermined	Undetermined	Undetermined			
17	14/07/2023	Station 4	Undetermined	Undetermined	Undetermined			
18	14/07/2023	Station 4	Undetermined	Undetermined	Undetermined			
19	14/07/2023	Station 5	Undetermined	Undetermined	Undetermined			
20	14/07/2023	Station 5	Undetermined	Undetermined	Undetermined			
21	31/08/2023	Station 1	32.47	33.52	32.78	943.68	460.59	764.86
22	31/08/2023	Station 1	33.13	33.2	33.78	602.11	572.39	385.24
23	31/08/2023	Station 2	Undetermined	Undetermined	Undetermined			
24	31/08/2023	Station 2	Undetermined	Undetermined	Undetermined			
25	31/08/2023	Station 3	Undetermined	Undetermined	Undetermined			
26	31/08/2023	Station 3	Undetermined	Undetermined	Undetermined			
27	31/08/2023	Station 4b	Undetermined	Undetermined	Undetermined			
28	31/08/2023	Station 4b	Undetermined	Undetermined	Undetermined			
29	30/09/2023	Station 1	Undetermined	Undetermined	Undetermined			
30	30/09/2023	Station 1	Undetermined	Undetermined	Undetermined			
31	30/09/2023	Station 2	Undetermined	36.25	Undetermined		3284.06	
32	30/09/2023	Station 2	Undetermined	Undetermined	Undetermined			
33	30/09/2023	Station 3	Undetermined	Undetermined	Undetermined			
34	30/09/2023	Station 3	Undetermined	Undetermined	Undetermined			
35	30/09/2023	Station 4b	Undetermined	Undetermined	Undetermined			
36	30/09/2023	Station 4b	Undetermined	Undetermined	Undetermined			
37	24/10/2023	Station 1	Undetermined	Undetermined	Undetermined			
38	24/10/2023	Station 1	Undetermined	Undetermined	Undetermined			
39	24/10/2023	Station 2	Undetermined	Undetermined	Undetermined			
40	24/10/2023	Station 2	Undetermined	Undetermined	Undetermined			

41	24/10/2023	Station 3	Undetermined	Undetermined	Undetermined
42	24/10/2023	Station 3	Undetermined	Undetermined	Undetermined
43	24/10/2023	Station 4b	Undetermined	Undetermined	Undetermined
44	24/10/2023	Station 4b	Undetermined	Undetermined	Undetermined

Note: Results from qPCR analysis of *T. bryosalmonae* are shown with Ct values, representing number of cycles required for the detection of the pathogen in each sample. Undetermined results show inconclusive results. Quantity shows amount of DNA copies measured each run.

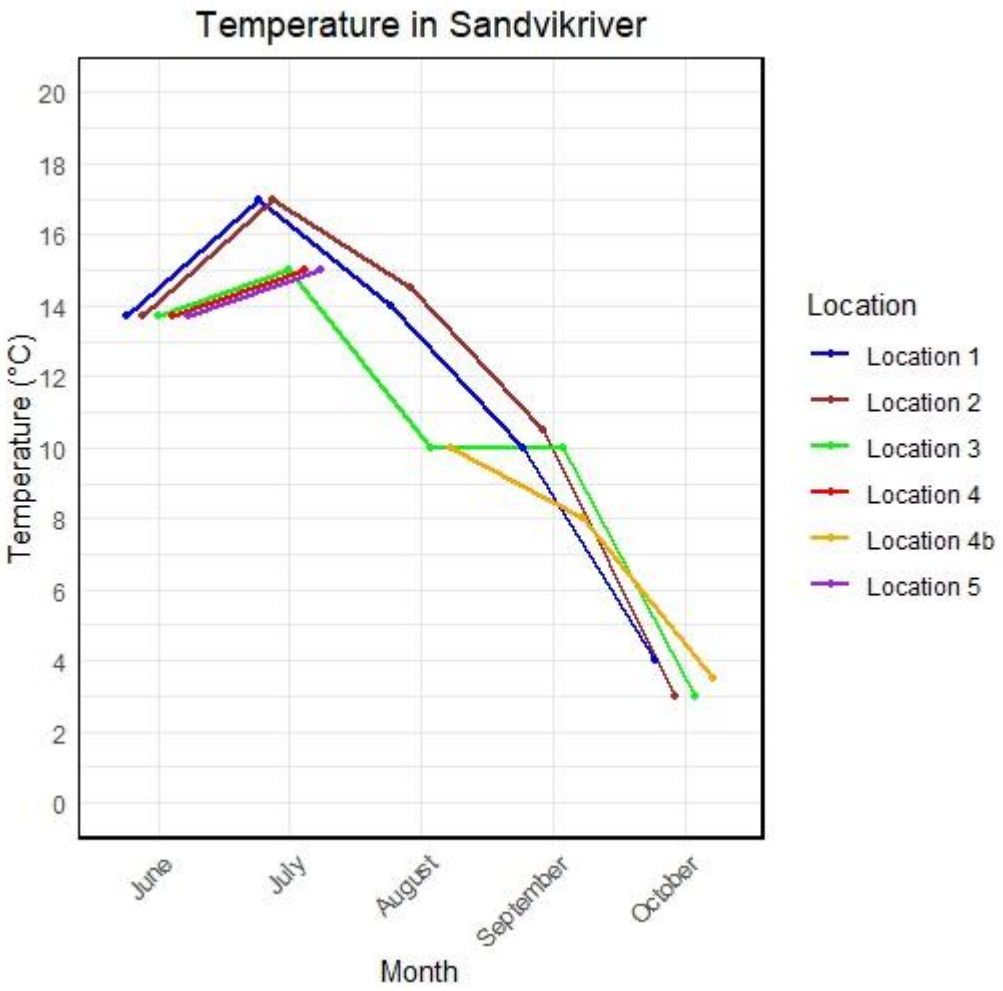

Figure A2. Temperature in River Sandvikselva

Figure A3. Construction notice